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## Limitations of Functional Recovery of Stretch Reflex Circuitry After Peripheral Nerve Regeneration

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**LIMITATIONS OF FUNCTIONAL RECOVERY OF STRETCH REFLEX  
CIRCUITRY AFTER PERIPHERAL NERVE REGENERATION**

**A dissertation submitted in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy**

**by**

**Gabrielle Marie Horstman  
B.A. The Ohio State University, 2005**

---

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Wright State University**

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WRIGHT STATE UNIVERSITY  
SCHOOL OF GRADUATE STUDIES

June 21, 2012

I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Gabrielle Marie Horstman ENTITLED Limitations of Functional Recovery of Stretch Reflex Circuitry After Peripheral Nerve Regeneration BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

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## ABSTRACT

Horstman, Gabrielle Marie. Ph.D., Biomedical Sciences Ph.D. Program, Wright State University, 2012. Limitations of Functional Recovery of Stretch Reflex Circuitry After Peripheral Nerve Regeneration.

Peripheral nerve regeneration fails to restore complete normal function after surgical repair of severed nerves, and this failure has primarily been attributed to errors in connecting with peripheral targets. However, recent evidence suggests that central deficits remain even after peripheral target reinnervation is largely successful. It has long been established that regeneration fails to restore the stretch reflex despite observation that many of the neural components are intact. Regenerated Ia afferents are largely successful in reinnervating muscle spindles, are capable of encoding stretch, and elicit EPSPs in homonymous motoneurons, while regenerated motor pools are capable of responding to uninjured sources of excitatory input. We now know this areflexia is due in part to a retraction of Ia afferent collaterals from motor pools in lamina IX (Alvarez et al., 2011). However, Ia afferents project not only to homonymous motoneurons but also to heteronymous synergist motor pools, even ones that are injury-spared. Ia afferents also project to antagonist motor pools through an interposed inhibitory interneuron. Therefore stretch of a muscle is capable of producing reflex contraction of both itself and synergist muscles while producing reflex inhibition of antagonist muscles. The function of these heteronymous projections after regeneration, however, remains unknown. The goal of this thesis is to determine the limitations of recovery of spinal circuit function after peripheral nerve regeneration by direct examination of stretch-evoked reflexes among synergists and antagonists. Direct

examination of the force response to stretch *in vivo* is extremely valuable as changes in behavior, the muscle response to stretch, after peripheral nerve regeneration must necessarily reflect changes in the underlying circuitry. We found that heteronymous stretch reflexes initiated by reinnervated muscle were dramatically decreased in both regenerated and injury-spared synergist motoneuronal pools. Additionally, both homonymous and heteronymous stretch reflexes were reduced in an injury-spared synergist after regeneration. These results give physiological evidence for retraction of regenerated Ia afferents from all synergist motor pools, and this retraction may extend to afferents that are injury-spared. Dysfunction also extends to antagonist stretch-evoked reflexes as we found a shift from net inhibition to net excitation of the injury-spared muscle due to reinnervated antagonist stretch. This shift is readily explained by the differential preservation of synapses located in lamina where interneurons mediating these responses are presumably located (Alvarez et al., 2011). Therefore functional deficits after peripheral nerve regeneration extend to heteronymous connections of Ia afferents with synergists and antagonists, both reinnervated and injury-spared. Taken together, these findings suggest that there is a profound discoordination of spinal reflexes and reorganization of spinal circuits after peripheral nerve regeneration.

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## **CHAPTER 1: General Background**

### **Introduction**

Peripheral nerves can be damaged in a number of ways. Traumatic injury to the nerve itself is a leading cause of peripheral nerve injuries in patients (Scholz et al., 2009) and can occur by severing the nerve completely or through compression. Additionally, numerous disease processes such as infection, autoimmune disorders such as that caused by systemic lupus erythematosus, and systemic diseases such as diabetes mellitus can also cause neuropathy. Regardless of the type of injury, damage to a tiny nerve out in the periphery sets off a myriad of changes within the central nervous system (CNS). These alterations range from molecular in scale to cellular and even to the circuit level. Furthermore, these changes are dramatic, uneven and widespread. For example, protein expression throughout the nervous system changes, inducing either up-regulation or down-regulation of various neuropeptides and cytokines (Fu and Gordon, 1997). An immune response occurs at the site of injury which plays a role in mediating Wallerian degeneration of the nerve distal to the trauma site (Stoll et al., 2002). However, immune responses also occur in the CNS near the cell bodies of injured neurons, affecting not only the injured neurons but also synaptic contacts it makes with neurons that are uninjured (Thams et al., 2008). Additionally, the normally tight blood brain barrier increases its permeability within 24 hours of sciatic nerve damage induced by either crush or cut (Beggs et al., 2010). Injured nerves also become hyperexcitable (Devor, 1995). But perhaps the most obvious change is that both the sensory input and the functional output mediated by the injured nerve is compromised. The loss of sensory input can lead to

numbness and pain as well as impairments in balance and coordination, while loss of motor output can lead to weakness, muscle atrophy, and fasciculations, with subsequent additional deficits in movement.

Fortunately, unlike in the CNS, nerves in the periphery have the ability to regenerate.

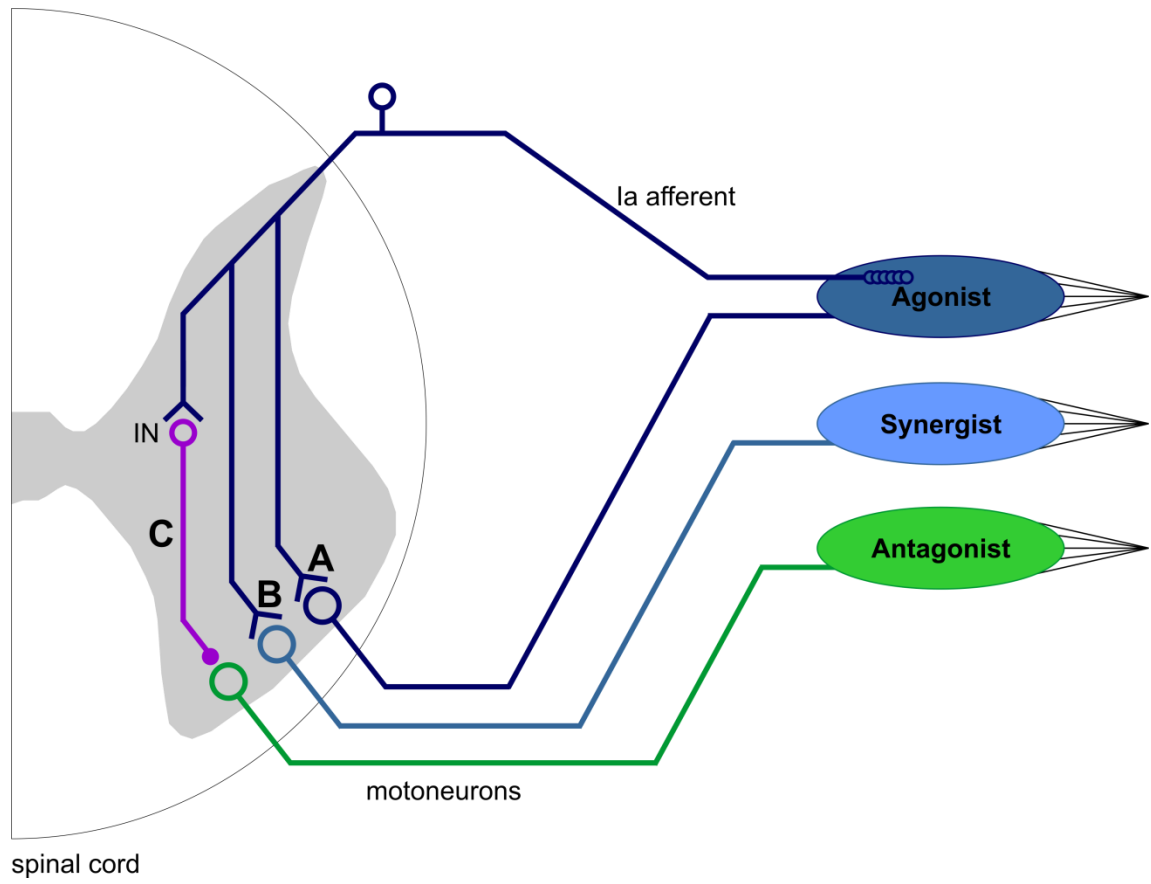
Therefore peripheral nerves that survive the initial injury have the capability of reconnecting to the CNS. The presumption is that reconnection of peripheral nerves will lead to recovery of all the changes that occur in response to injury. Certainly some of these changes are reversed. For example, changes in intrinsic electrical properties which underlie hyperexcitability in motoneurons after axotomy return to pre-axotomy levels once regeneration has occurred (Bichler et al., 1997; Bullinger et al., 2011a; Foehring et al., 1986a; Mendell et al., 1995; Nakanishi et al., 2003). Once motoneurons reconnect with muscle fibers, over time muscle atrophy reverses and weight returns to normal. Muscle tension and force output also return to normal levels (Cope and Clark, 1993; Cope et al., 1994; Foehring et al., 1986a). Despite these successes, peripheral nerve regeneration is insufficient in restoring full recovery on both cellular and functional levels. For example, it appears that the expression of specific neurotransmitter transporters in motoneurons is permanently lost (Alvarez et al., 2011). From a functional standpoint, peripheral nerve regeneration fails to restore complete normal function in 90% of patients who have undergone surgical repair of median or ulnar nerves. Specifically, these patients are left with persistent deficits in fine motor control and tactile discrimination. Furthermore, 40% of patients fail to regain any useful function whatsoever (Brushart, 2011). Given these observations, it is clear that a more detailed understanding of the apparent limitations on functional recovery after peripheral nerve regeneration is necessary in order to expand the knowledge of just how widespread these limitations are.

This inability of peripheral nerve regeneration to restore complete normal function has primarily been attributed to peripheral deficits. Errors in reconnection of axons with their appropriate peripheral targets leads to a misdirection of sensory and motor information carried between the CNS and periphery (Brushart, 2011; Sunderland, 1978). However, recent evidence obtained in studies investigating stretch areflexia after peripheral nerve regeneration suggests that central deficits remain even after peripheral target reinnervation is largely successful. The failure of peripheral nerve regeneration to restore the stretch reflex was first observed and investigated in the early nineties (Cope and Clark, 1993; Cope et al., 1994), whereby stretch of a muscle fails to produce a reflex contraction in that same muscle. This is despite observation that approximately 50% of regenerated sensory afferents responsible for conveying information about muscle length to the CNS (primary muscle spindle afferents) successfully reinnervate the mechanoreceptors responsible for detecting changes in muscle length, muscle spindles (Collins et al., 1986), and that regenerated motoneurons are capable of firing in response to excitatory input (Cope and Clark, 1993; Cope et al., 1994). We now know that synapses between primary muscle spindle afferents and motoneurons are permanently lost anatomically after peripheral nerve regeneration. Alvarez et al. (2011) found that the expression of vesicular glutamate transporter 1 (VGLUT1), a common marker for primary muscle spindle afferents (Alvarez et al., 2004), was decreased by 75% on the cell body of motoneurons. This loss was explained by the demonstration that primary muscle spindle afferents physically retract from lamina IX in the spinal cord where motoneuron cell bodies are located. Anatomical retraction was subsequently confirmed physiologically by Bullinger et al. (2011a). The authors demonstrated that individual afferents that do respond to stretch fail to generate excitatory post-synaptic potentials (EPSPs) greater than 80% of the time in regenerated motoneurons.

Additionally, there was a 40% decrease in afferent ability to generate aggregate excitatory stretch-evoked synaptic potentials (eSSPs) in motoneurons. In cases where eSSPs were generated, the amplitude was reduced by half. Therefore even though errors in peripheral connections certainly play a role in stretch areflexia, neurons that do make the proper peripheral connections are still centrally impaired.

It is this central impairment that leads us to question the degree to which functional recovery of spinal circuits is limited after peripheral nerve regeneration. We know that homonymous stretch reflexes are not restored. However, primary afferents mediating the stretch reflex send multiple projections throughout the spinal cord (Figure 1.1). In particular they make connections not only with homonymous motoneurons (motoneurons innervating the same muscle, Figure 1.1A) but also with heteronymous motoneurons (motoneurons innervating different muscles) of close synergists (Figure 1.1B). Furthermore, these stretch sensitive primary afferents make connections with antagonist motoneurons through an interposed inhibitory interneuron (Figure 1.1C). This pathway is known as reciprocal inhibition, where stretch of a muscle inhibits its antagonist muscle. Additionally, this central impairment may extend to injury-spared stretch-activated circuits both homonymous and heteronymous in their projection, even in cases where injury-spared synergists are in a position to compensate for lost feedback. The functionality of these afferent projections after peripheral nerve regeneration, both regenerated and injury-spared, remains relatively unknown, although there have been hints they may also be disrupted (Cope et al., 1994). Using the stretch reflex as a base model, the extent of recovery of spinal circuit function after peripheral nerve regeneration can be studied by direct examination of stretch-evoked reflexes among synergists and antagonists.

**Figure 1.1. *Projections of Ia afferents mediating stretch-evoked reflexes.*** Stretch reflex (A&B): Muscle stretch in an agonist activates Ia afferents which project monosynaptically to homonymous motoneurons (A) as well as to heteronymous motoneurons of synergist muscles (B) in order to elicit reflex contraction. Reciprocal Inhibition (C): Muscle stretch of an agonist activates Ia afferents which also project disynaptically to antagonist muscles to inhibit muscle contraction through Ia inhibitory interneurons (IN). Excitatory connections are represented by open forks while inhibitory connections are represented by filled circles..





Use of the stretch-evoked reflexes to study the limitations of recovery of spinal circuit function after peripheral nerve regeneration is beneficial for a number of reasons. First described by Liddell and Sherrington (1924), the monosynaptic stretch reflex circuit in particular is one of the best-characterized circuits in the nervous system and as such has been used extensively as a model for neural plasticity after nerve injury. In its most basic form, the stretch reflex consists of a two-neuron circuit where stretch information is carried centrally into the spinal cord by primary Ia afferents where they synapse directly with motoneurons. Sufficient depolarization of motoneurons then causes a reflex contraction in muscle. Due to this simplicity, it is easy to see how a given input, in this case muscle stretch, results in a direct output, muscle contraction. If motoneurons fire, there is an obligatory contraction of muscle fibers. Therefore there is good correspondence between the circuit and the output of muscle force. This functional output makes this circuit an especially powerful tool as it is directly linked to a measurable behavior. The amount of force a muscle produces due to muscle stretch can be measured and quantified. In the case of heteronymous stretch reflexes, increases in muscle force would indicate facilitation, while decreases in muscle force would indicate inhibition. The ability to examine the muscle force response to stretch is extremely valuable. First, it enables us to study the response of the net operation of a population of motoneurons as a whole. Second, by studying changes in the response of muscle to stretch we can infer changes in the spinal circuitry underlying those actions. Furthermore, these measurements can be made using an *in vivo* preparation. Therefore experiments using this design have direct physiological relevance since neural circuit behavior can be inferred from a direct functional behavior in a living, breathing animal.

The studies presented in this work will be the first to provide direct detailed physiological evidence for the status of stretch-evoked reflexes among synergists and antagonists after

peripheral nerve regeneration. These studies will not only expand knowledge of the limitations of spinal circuit recovery but will also provide valuable insight about the reorganization of spinal circuits after peripheral nerve regeneration.

### **Neural Response to Muscle Stretch**

By monitoring muscle stretch, the nervous system can detect limb disturbances and respond in a way which returns the limb to its intended position. This is accomplished through the stretch reflex and is the body's fastest response to unexpected perturbations. For example, a person is standing still and suddenly experiences a push from behind. If nothing is done to compensate for this disturbance, the person may continue to fall forward. Luckily, the nervous system generates a coordinated response in order to return the body to the upright position. The forward push stretches the achilles tendon which elicits contraction in the triceps surae muscles (ankle extensors). The resulting contraction of the triceps surae then assists in restoring leg position so the person remains upright. However, stretch of the achilles tendon also inhibits ankle flexors, such as the tibialis anterior (TA). This allows for ankle extensor contraction to occur unimpeded which increases the efficiency and expediency of the corrective response.

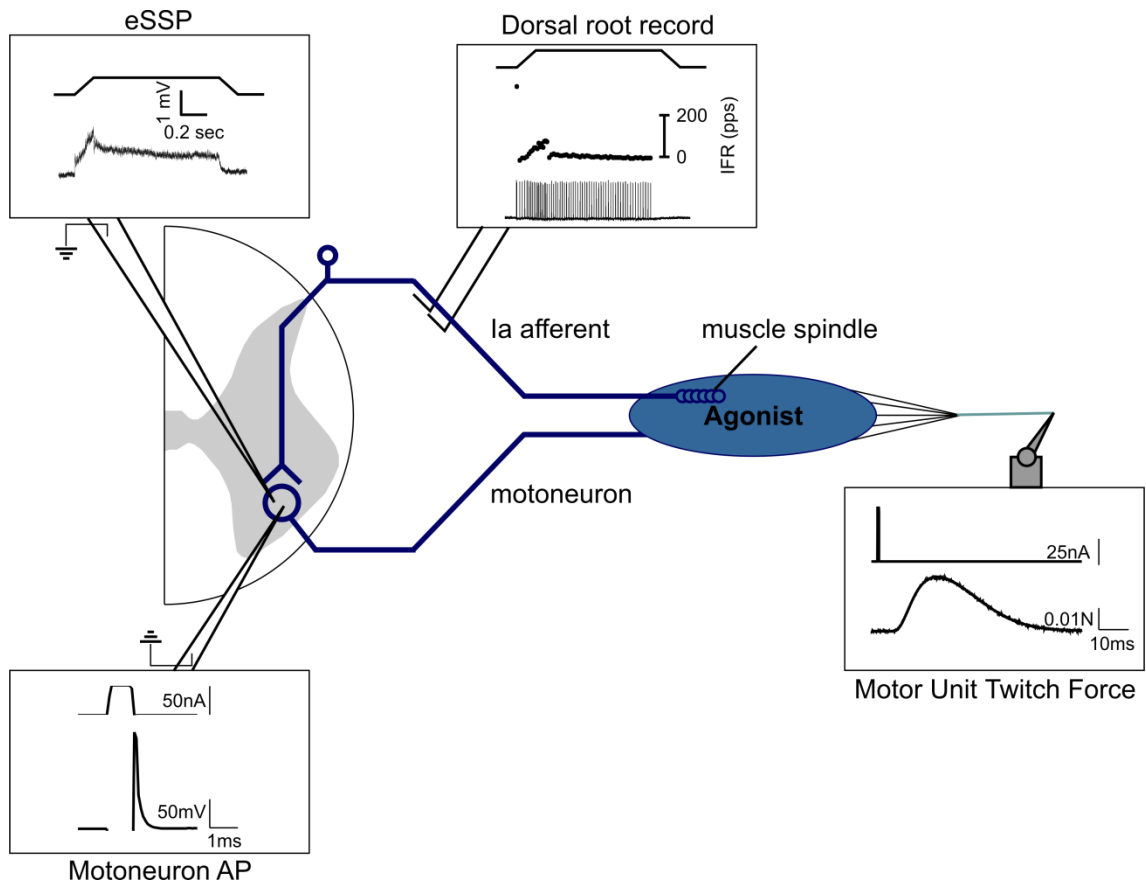
These actions are mediated primarily through the divergent connections of spindle afferents at the segmental level. Primary spindle afferents of a stretched muscle activate both homonymous and synergistic motor pools through monosynaptic excitatory connections. They also inhibit antagonistic motor pools through disynaptic pathways through via premotor inhibitory interneurons. This divergence ensures that the appropriate combination of synergists will be activated and the appropriate antagonist muscles are inhibited. Furthermore, it ensures that muscles acting around a joint do not act independently of each

other. In other words, muscles with similar and opposing actions at a joint work together as a unit. The system of reflex pathways underlying these actions is referred to as the myotatic unit and was first described by Lloyd in 1946. The myotatic unit therefore coordinates the activity of individual synergist and antagonist muscles around a joint in a manner that opposes stretch. Although further examination revealed that this organization may be distributed as to facilitate actions around a joint rather than strict agonist-antagonist pairings, (Eccles et al., 1956; Eccles and Lundberg, 1958), one thing remains clear: the organization of stretch feedback allows for the body to respond to unexpected perturbations in order to maintain balance. The neural circuitry mediating this coordinated response will be described next.

### **Neural Circuitry Underlying Stretch-Evoked Reflexes**

In order to understand the limitations of functional recovery of stretch-evoked reflexes after peripheral nerve regeneration, knowledge of the components and normal operation of the circuitry are essential. This thesis is concerned with the circuitry underlying two stretch-evoked reflexes: the monosynaptic stretch reflex and reciprocal inhibition. The circuitry for the monosynaptic homonymous stretch reflex circuit will be described first and is shown in Figure 1.2. Muscle spindles are activated in response to muscle stretch. The resulting action potentials are propagated along the axons of primary group Ia muscle afferents to their central terminals in the spinal cord where they make monosynaptic connections with motoneurons. Stretch-evoked excitatory post-synaptic potentials (eSSPs) are produced in the motoneurons through the process of chemical transmission. When the motoneuron becomes sufficiently depolarized, action potentials are propagated down the motor axon to the interface between the motor axon and the extrafusal muscle fibers, the neuromuscular junction. Chemical transmission again ensues, the muscle fiber contracts via excitation-

**Figure 1.2. *Components of the monosynaptic stretch reflex.*** Muscle stretch is sensed by muscle spindles. This information is then encoded and transmitted to the spinal cord via Ia afferents (Dorsal root record box) and synapse directly with alpha motoneurons. Excitatory stretch-evoked synaptic potentials (eSSPs) can be recorded in motoneurons when the muscle is stretched (eSSP box). Activation of individual motoneurons (Motoneuron AP box; AP: action potential) causes extrafusal muscle fibers to contract (Motor Unit Twitch Force box). Data in each box is taken from our lab. Dorsal root record: extracellular recording of a single afferent in response to ramp hold release stretch. eSSP: excitatory stretch-evoked synaptic potential that results from Ia afferent encoding shown in Dorsal root record. Motoneuron AP: Action potential evoked in a motoneuron due to intracellular current injection. Motor Unit Twitch Force: Amount of force produced by a single motor unit in response to the action potential generated due to intracellular current injection.



contraction coupling, and the reflex is complete. Given this arrangement it is easy to see how successful output of this circuit, generation of muscle force, depends on each component working properly. In the case of reciprocal inhibition where Ia afferents mediate inhibition of antagonists, there is a single inhibitory interneuron interposed between the Ia afferent and an antagonist motoneuron. Initially, the pathway is activated in the same manner as the monosynaptic stretch reflex. However, Ia afferents send off branches which synapse directly with premotor inhibitory interneurons (INs). These inhibitory INs in turn synapse with antagonist motoneurons. Activation of premotor inhibitory INs produces inhibitory post synaptic potentials (IPSPs). This causes the antagonist motoneuron to hyperpolarize which inhibits action potential generation and, consequently, muscle contraction.

### ***Components of Stretch-Evoked Reflexes***

The stretch-evoked reflexes central to this thesis all share the same basic components: muscle spindles, Ia afferents, motoneurons, and muscle fibers. In the case of Ia projections to antagonists through reciprocal inhibition, there is the addition of a premotor inhibitory IN. Certainly this circuit is influenced by descending inputs, but they will not be considered here as the experimental design minimizes their contribution.

### **Muscle Spindles**

Changes in muscle length are sensed by mechanoreceptive muscle spindles. The majority of our understanding of the muscle spindle apparatus comes from initial studies by Sherrington (1894) and Ruffini (1898), Barker (1948), Boyd (1972), and Matthews (1933). The spindle lies within the muscle mass and is arranged in parallel with extrafusal muscle fibers. It consists of two types of small, encapsulated intrafusal muscle fibers, called chain and bag fibers respectively, which are innervated by both afferent and efferent nerves. Increased or

decreased activation of efferent endings causes the intrafusal muscle fibers to contract by varying degrees, thereby controlling the sensitivity of the spindle apparatus. Afferents are arranged in two specific arrangements, annulospiral rings and flowerspray patterns, which correspond to primary and secondary afferents, respectively. The unique arrangements of these primary and secondary endings within the spindle apparatus are thought to account for their specific encoding properties. Changes in the length of intrafusal muscle fibers (due to lengthening of the muscle itself) cause these afferent endings to depolarize through mechanosensitive ion channels (Simon et al., 2010). In this manner information about muscle length is encoded for transmission to the CNS. In the cat, there are approximately 35, 62, and 56 muscle spindles in the LG, MG, and SOL, respectively (Chin et al., 1962), and approximately 71 spindles in the TA (Barker and Chin, 1960).

### Muscle Spindle Afferents

Information about muscle length sensed by muscles spindles is encoded and transmitted to the CNS though both primary group Ia afferents as well as secondary group II afferents (Bradley and Eccles 1953, Hunt 1954, Matthews 1933, Matthews 1972). Ia afferents are thought to innervate primary endings of muscles spindles, while group II afferents innervate the secondary endings. While this holds true in the cat, it remains a point of discussion in the rat (De-Doncker et al., 2003). There is only a single Ia afferent per muscle spindle, while group IIs range in number from 0-5 (Barker 1962; Boyd 1962). Afferent classification is based on axonal diameter, conduction velocity, and firing characteristics. In the cat, group I afferents have axon diameters from 12-20um and conduction velocities between 72-120 m/s. Group II afferents have smaller axon diameters from 4-12um and resulting slower conduction velocities from 20-70m/s (Matthews 1972). In the rat, however, there is considerable overlap in the conduction velocity ranges between these afferents (Lawson and Waddell, 1991).

While both Ia and group II afferents fire in response to length changes, Ia afferents in particular exhibit greater responses to changes in muscle stretch velocity than group II afferents and as such are able to detect the rate of muscle stretch. They are exquisitely sensitive to high frequency vibration, whereas group IIs are not as sensitive (De-Doncker et al., 2003; Matthews, 1972). While the afferent cell bodies are located in the dorsal root ganglia outside of the CNS, their axons send multiple projections segmentally throughout the spinal cord as well as through ascending tracks to supraspinal centers. The synapse made by Ia afferents with motoneurons is glutamatergic (Jahr and Yoshioka, 1986).

#### Premotor Inhibitory Interneurons

In the case of reciprocal inhibition between antagonist muscles, a premotor inhibitory interneuron is interposed between the Ia afferent and the motoneuron. These interneurons themselves receive input from a variety of segmental and descending inputs (e.g. Baldissera et al., 1981; Jankowska 1992). However, the only monosynaptic proprioceptive input they receive is from Ia afferents (Hultborn et al., 1971b, 1976). These premotor inhibitory interneurons are located in the ventral part of lamina VII in the spinal cord (Hultborn et al., 1976b; Jankowska and Lindström, 1972). Strong physiological evidence obtained after administration of strychnine, a glycine antagonist (Johnston, 1978) suggests that the synapse between premotor inhibitory INs and antagonist motoneurons is glycinergic as IPSPs were suppressed (Bradley et al., 1953; Eccles et al., 1954; Lodge et al., 1977). Interestingly, these inhibitory interneurons are the only segmental interneurons found to receive recurrent inhibition from Renshaw cells (Hultborn et al., 1971a).



## Alpha Motoneuron

Alpha motoneurons (motoneurons) are spinal motoneurons that innervate the extrafusal muscle fibers of skeletal muscle. Their cell bodies are located in lamina IX of the spinal cord and are arranged in topographic pools according to their target muscle (Brown and Fyffe, 1981; Munson et al., 1982). Motoneurons innervating the cat hindlimb have large, relatively symmetric, radially organized dendritic trees which receive approximately 50,000 synapses from both segmental and descending sources (Fyffe 2001). Motoneuron firing properties are determined by a combination of non-uniformly distributed active and passive properties of the dendrites, corresponding to voltage- and calcium-dependent channels and dendritic electrotonic structure, respectively (Fyffe 2001). Therefore motoneurons are the final common pathway for nervous system output as they are the last point of integration of neural inputs before muscle fibers are activated. Motoneurons can be classified on the basis of the type of muscle fiber they innervate (see below).

## Skeletal Muscle

Extrafusal muscle fibers (skeletal muscle fibers) are responsible for muscle contraction and corresponding force output. Each muscle fiber is composed of a bundle of tubular muscle cells called myofibrils. These in turn are composed of repeating sections of sarcomeres containing both thick and thin filaments on which are located the key contractile proteins myosin and actin, respectively. When an action potential is generated in a motoneuron, it propagates down the motor axon to the synapse between it and the muscle fiber, the neuromuscular junction. Acetylcholine is released and causes a depolarization of the endplates of the muscle fiber. This depolarization results in the release of calcium which in turn activates the process of ATP-dependent excitation-contraction coupling, mediated by actin and myosin. Myosin hydrolysis of ATP primes myosin, allowing for its globular head

to to form a cross bridge with actin on the thin filament. Once attached, the release ATP hydrolysis products induces a conformational change which causes the thin filament to slide over the thick filament. These cross bridges between actin and myosin are formed, released, and reformed in a cyclic manner with subsequent sarcomere shortening, thus allowing for muscle contraction. There are three classes of extrafusal muscle fiber, based on histochemical and physiological analysis: slow twitch (slow), fast oxidative glycolytic (fast fatigue-resistant), and fast glycolytic (fast fatiguing; Lieber, 2002). A single motoneuron innervates several muscle fibers, and each fiber is innervated by a single motoneuron; therefore a motoneuron and the fibers it innervates are referred to as a motor unit. Thus when a motoneuron fires an action potential, the muscle fibers it innervates contract without fail. Motor units are activated according to the Henneman size principle, where small motoneurons are more readily activated and are therefore recruited before larger ones (Henneman et al 1965). Increases in both motor unit recruitment and firing rate increase the amount of force produced by muscle (Monster and Chan, 1977).

### **Use of Muscle Force as a Tool for Studying Stretch Reflex Circuit Function**

The stretch reflex circuit is unique in that its functional output is highly accessible. It is possible, therefore, to assess the status of the neural circuitry underlying the stretch reflex by studying the muscle force output in response to controlled stretch. The force response to stretch is representative of the net operation of a pool of motoneurons, where motoneuron activation necessarily causes muscle fiber contraction. If the circuitry is functioning normally, force response to stretch would remain unchanged. However, disruptions of any portion of the circuitry would be expected to interfere with the ability of stretch to elicit force responses, resulting in responses that are either decreased or completely absent.

There are three main components to the force response to stretch (Nichols and Houk, 1976). One is a purely passive, mechanical response called the passive intrinsic response. This response is almost entirely due to the viscoelastic properties of muscle, with a small contribution from low level cross bridge cycling (Hill 1968; Hoffer and Andreassen, 1981). These properties are what determine the stiffness of a muscle when it is not actively contracting. The passive intrinsic response can be easily obtained by recording the force response to stretch of a muscle whose nerve has been cut. The active intrinsic component accounts for the increased muscular stiffness during contraction. The force amplitude of the active intrinsic response is dependent on background force, and this relationship is linear (Nichols and Houk, 1976; Hoffer and Andreassen, 1981; Huyghes-Despointes et al., 2003a). This can be explained as such: as more motor units become active, more cross bridges are formed and undergo cycling, thus increasing the stiffness of the muscle. The last component of the force response to stretch, the reflex response, is attributable entirely to neural reflex action. During muscle lengthening, the reflex component is large while the mechanical component is small. This is due to a yielding of the mechanical response with muscle stretch. With dorsal roots rhizotomy effectively abolishing reflex input, Nichols and Houk (1976) demonstrated that the mechanical response to ramp hold release muscle stretch yielded abruptly within 60µm of muscle stretch, similar to results obtained with ventral root rhizotomy and stimulation (Joyce et al., 1969). Furthermore, when compared to the force response to stretch before dorsal root rhizotomy, the reflexive and mechanical force traces overlapped during this brief timeframe. This short-range stiffness of the muscle is thought to be due to the slippage of long-term stable cross bridges in resting muscle being pulled apart by muscle stretch. The ability of these cross bridges to bend and stretch before slippage is also thought to contribute to the initial resistance of muscle to stretch (Hill, 1968). The mechanical properties of elastic components of the contractile apparatus, such as titin, are

also believed to contribute to this short range stiffness (see Horowitz 1999 for review). Both intrinsic and reflexive components of the force response to stretch can be affected by prior movement. Huyghues-Despointes et al. (2003b) demonstrated that prior muscle shortening increases the active mechanical component and decreases the reflex component.

It stands to reason that if the response of a muscle to stretch of itself is greater than the mechanical response, the neural reflex action is necessarily dominated, at least initially, by excitation. Since the initial stimulus for this excitation is stretch, it seems highly likely that muscle spindles are the dominant receptor component, and therefore spindle afferents are indeed primarily responsible for motoneuron excitation. The earliest excitatory response to muscle stretch occurs around 16ms, indicating that rapid excitatory spinal reflexes should be involved (Nichols, 1999). This delay implicates both Ia and group II afferent inputs, both of which synapse monosynaptically with homonymous motoneurons. However, the inputs from group IIs are relatively weak (Prochazka, 1990). Therefore although the contribution of group II afferent excitation in generating this early force response to stretch cannot be excluded, it is more likely that Ia afferents are the predominant source of excitation. The Ia afferent firing profile also mimics the profile of the force output attributable to reflex action (Houk and Rymer, 1981). Both maximal afferent discharge and the maximal reflex force occur during the dynamic phase of stretch. The dynamic phase of stretch corresponds to the initial length change due to stretch, and is often measured as a single point at the end of the ramp. These observations also correlate with the maximal amount of EMG occurring during ramp hold release stretch. The contribution of Ia afferents to motor force output in response to stretch was further examined in studies where stretch was superimposed upon a tonic vibration reflex (Houk et al., 1981; Jack and Roberts, 1978). Since Ia afferents are exquisitely sensitive to muscle vibration, the tonic vibration reflex effectively clamps their discharge,

making them relatively unresponsive to a superimposed stretch. When these responses are compared to the normal mechanical and reflex responses during ramp hold release stretch at matched background forces, the amount of initial dynamic force is suppressed almost entirely. Again, this suggests that the initial dynamic component of the force response to stretch is due to short latency Ia stretch reflexes. It should be noted that Ia afferents also make polysynaptic, excitatory connections with homonymous motoneurons (Baldissera et al., 1981) and would thus be expected to be involved in the force response in some way. Unfortunately, polysynaptic versus monosynaptic contributions to the force response to stretch are not well documented. Muscle reflex interactions between synergist and antagonist muscles also suggest that the stretch evoked reflexes mirror the projections of Ia afferents studied intracellularly, further supporting the conclusion that Ia afferents play a dominant role in the force response to stretch. These interactions will be discussed below.

### **Synaptic Study of Stretch-Evoked Reflexes**

With the individual components discussed, we now turn our attention to the physiology of the circuitry underlying stretch-evoked reflexes. The Ia-motoneuron synapse is one of the best-characterized synapses in the mammalian nervous system and has been extensively used as a model for neural plasticity after injury and regeneration. Early intracellular studies of Ia projections were later supplemented by direct investigations of muscle force response to stretch, with the findings of these two very different physiological techniques often complimenting one another. The normal circuit function mediated by the Ia afferent projections discussed here is imperative to the identification of changes after peripheral nerve injury.

### ***Physiological Study of Ia Afferent Projections to Homonymous Motor Pools***

Electrical stimulation of peripheral nerves at levels sufficient to activate Ia afferents generates an excitatory post-synaptic potential (EPSP) which can be recorded in homonymous motoneurons with monosynaptic latencies (Eccles et al., 1952; 1957a; cg Figure 1.1A). Although they are largely influenced by Ia input, it is likely that monosynaptic group II inputs as well as polysynaptic inputs from Ib afferents (primary afferents responsible for transmitting force feedback) contribute to EPSPs as well (Cope et al., 2001). However, stimulation of individual physiologically identified Ia afferents generate homonymous EPSPs on their own. Furthermore, study of individual Ia afferents demonstrated that all Ia afferents in a single muscle project to nearly all homonymous motoneurons. For example, all 60 Ia afferents in the cat MG make homonymous synapses with almost all 300 MG motoneurons present (Mendell and Henneman, 1971). More physiologically relevant stimulation has also been used to study the Ia-motoneuron synapse. Excitatory stretch-evoked synaptic potentials (eSSPs) can be evoked in homonymous motoneurons by small triangular stretch (Stuart et al., 1971), ramp hold release stretch, and vibration of muscle (Westbury, 1972). The amount of depolarization varies with the parameters of stretch, and this depolarization tends to be larger in motoneurons innervating slow twitch motor units than those innervating fast twitch motor units. Furthermore, muscle stretch always produces eSSPs in the corresponding motoneurons in control animals (Bullinger et al., 2011a; Haftel et al., 2005). The observation that the shape of eSSPs closely resembles the firing profile in physiologically characterized Ia afferents evoked by muscle stretch implies that eSSPs are generated primarily by spindle afferent inputs to motoneurons (Bullinger et al., 2011a).

### ***Physiological Study of Ia Afferent Projections to Synergist Motor Pools***

Stretch reflexes are not restricted to homonymous muscles. This is because Ia afferents diverge to make monosynaptic heteronymous connections with synergistic motoneuron pools (Figure 1.1B). This divergence therefore amplifies the net effect of stretch feedback from a single muscle, ensuring that muscles with similar actions at a given joint work together. Electrical activation of Ia afferents produces EPSPs in synergist motoneurons at monosynaptic latencies (Eccles et al., 1957a; Scott and Mendell, 1976). The projection of Ia afferents among the triceps surae motor pools, consisting of motoneurons innervating the medial (MG) and lateral (LG) gastrocnemius and soleus (SOL) muscles, in particular has been well documented. For example, Ia afferents from the MG project to nearly 2/3 of the approximately 450 LG & SOL motoneurons (Prochazka, 1990). Therefore although Ia afferents project to nearly all homonymous motoneurons, they project to a smaller portion of the motoneurons belonging to synergists (Scott and Mendell, 1976). In general, EPSPs generated in heteronymous motoneurons have a smaller amplitude than those generated in homonymous motoneurons and has been demonstrated specifically for the ability of LGS (LG and SOL together) to elicit EPSPs in MG motoneurons (Burke et al., 1976; Scott and Mendell, 1976). It should be noted, however, that individual EPSPs generated in heteronymous motoneurons from single afferents are similar in size to those generated in homonymous motoneurons when differences in motoneuron size and position are minimized, (Webb and Cope, 1992). Small, brief triangular stretch of muscle has been shown to elicit eSSPs in heteronymous motoneurons (Stuart et al., 1971). MG and LGS motoneurons were antidromically identified and SSPs were recorded in response to either MG or SOL stretch. Unfortunately, little information is available on the comparability in eSSP size between homonymous and heteronymous SSPs amongst the triceps surae.

These electrophysiological studies have been supported by in vivo experiments in the cat in which the contractile force output in response to stretch of a muscle was measured directly. By stretching two synergists simultaneously, increased excitatory drive from stretch of one muscle would be expected to increase the contractile response in a synergist to a force greater than its own homonymous stretch reflex. Stretch of any of the triceps surae simultaneously with another triceps surae muscle produces not only a homonymous stretch reflex in itself but also a heteronymous stretch reflex in its synergist (Nichols 1989, Nichols 1999). Furthermore, the increased reflex force in response to homonymous stretch was greater than the additional force generated by heteronymous stretch. This suggests that synaptic excitation is stronger from homonymous muscles than from heteronymous muscles. The relative strengths of heteronymous reflex force when the triceps surae muscles are interacted by stretch also mirrors the relative strengths of the heteronymous EPSPs generated amongst each other (Eccles et al., 1957a; Nichols 1999). For example, heteronymous reflexes from MG and SOL onto LG tended to be strongest, while heteronymous reflexes tended to be weakest from SOL and LG onto MG.

### ***Physiological Study of Ia Afferent Projections to Antagonist Motor Pools (Reciprocal Inhibition)***

The pattern of reciprocal inhibition was first described by Lloyd (1946) and further investigated by Laporte and Lloyd (1952) where inhibition of antagonists was documented by studying the ability of afferent volleys to condition ventral root responses. It wasn't until the introduction of intracellular recording techniques that electrical stimulation of Ia afferents was found to produce IPSPs at disynaptic latencies in antagonist motoneurons (Araki et al., 1960; Eccles et al., 1956; Eccles and Lundberg, 1958; Jankowska and Roberts, 1972). The IPSPs produced in the antagonist motoneurons may also be larger in flexor than in extensor



motoneurons (Eccles and Lundberg, 1958). The Ia inhibitory INs involved were also found to be a site of convergence for Ia input from synergist muscles acting collectively to inhibit the antagonist (Hultborn and Udo, 1972). Initial investigations of stretch-evoked synaptic responses in motoneurons by Granit et al (1964) also demonstrated inhibitory interactions between antagonist muscles. Stretch of the TA elicited hyperpolarization of popliteal motoneurons, which would include motoneurons belonging to the triceps surae. Conversely, stretch of the triceps surae elicited hyperpolarization of peroneal motoneurons, which would include motoneurons belonging to TA. Inhibitory SSPs (iSSPs) have also been demonstrated in TA-EDL (extensor digitorum longus) motoneurons in response to small, brief triangular stretch of the MG (Watt et al., 1976).

The inhibition of antagonist muscles was also demonstrated through studies of muscle reflex interactions of the type described above. Nichols (1989) demonstrated that stretch of the tibialis anterior (TA, ankle flexor) inhibited the homonymous force response to stretch in all three triceps surae muscles (ankle extensors), with the largest amount of inhibition seen from TA to SOL. The MG, on the other hand, demonstrated the largest amount of inhibition onto the TA with little inhibition coming from LG or SOL. Further study of the ability of TA to inhibit SOL (Nichols and Koffler-Smulevitz, 1991) suggests that the inhibition seen between antagonists during simultaneous stretch is at least in part mediated by reciprocal inhibition. First, the amount of inhibition was shown to be length dependent. Second, injection of intravenous strychnine was shown to block this inhibition while other inhibitory neurotransmitter antagonists did not.

### **Other Afferents Activated During Muscle Stretch**

Ia afferents are not the only afferents that fire during muscle stretch. Ib afferents, which normally transmit information about muscle force to the CNS, are also activated during stretch. At rest, Ib afferents have been well-documented in mediating di- or trisynaptic, inhibitory pathways, termed non-reciprocal group I inhibition (Eccles et al., 1957b; Granit, 1950; Granit and Surrsoet, 1949; Hunt 1952; Laporte and Lloyd, 1952; Sherrington, 1909). These observations have been supported by direct physiological study of muscle force responses in the presence or absence of background reflex force for both homonymous (Jack et al., 1989) and heteronymous (Bonasera and Nichols, 1994, 1996; Nichols, 1989; Wilmlink and Nichols, 2003) pathways. Additionally, Ia afferents have been shown to contribute to non-reciprocal inhibition, although the strength of this inhibition is much weaker than inhibition from Ib's (Fetz et al., 1979). Ib afferents also mediate facilitation in non-locomoting preparations, especially from extensors onto flexors although the inverse has also been observed (Laporte and Lloyd, 1952; Eccles et al., 1957b). The distinction of a non-locomotor preparation is important as a switch from an inhibitory Ib pathway to one of excitation has been observed during fictive locomotion (for review see Lam and Pearson, 2002), although subsequent study in spontaneously locomoting preparations has shown that this phenomenon may be limited to specific muscles (Ross and Nichols, 2009).

### ***Ib Afferents and Their Response to Different Stimuli***

Primary group Ib afferents are proprioceptive afferents that are responsible for transmitting information about muscle force to the CNS (Laporte and Lloyd 1952, Hunt 1952). They innervate the mechanoreceptive Golgi tendon organs (GTOs) whose adequate stimulus is muscle contraction and are capable of sensing the contraction of single motor units (Houk and Henneman 1967). Each GTO is usually innervated by a single Ib afferent (Jami, 1992).

Ib afferents are one of the two primary (group I) proprioceptive afferents, the other being Ia afferents, and as such have similar axon diameters and conduction velocities. However, there seems to be fewer Ib afferents in the upper range than Ia's (Jami, 1992). Ib afferents appear to be more sensitive to changes in muscle force (e.g. dynamic sensitivity to muscle force) than the level of force (Horcholle-Bossavit et al., 1990). Nevertheless, Ib afferents are also excited by steady stretch of the muscle. They respond to both ramp-hold-release and triangular stretch (Stuart et al., 1970; Bullinger et al., 2011b), but are not sensitive to high frequency stretch (vibration) as are Ia afferents (Matthews, 1933). This difference can be exploited in order to selectively activate one or the other (Coppin et al., 1970; Jack and Roberts, 1978; Jankowska, 1982).

### ***Projections of Ib Afferents and Location of Target Interneurons***

Upon entering the spinal cord, Ib afferents primarily send off collaterals in lamina V-VII in the dorsal horn of the spinal cord (Brown and Fyffe 1979) where they form synapses with various interneurons. The interneurons mediating non-reciprocal inhibition are located in lamina V-VI (Czarkowska et al., 1981; Jankowska et al., 1981). Excitatory interneurons that receive input from Ib afferents are similarly located (Bannatyne et al., 2006; Bannatyne et al., 2009; Jankowska et al., 2009). However, the projections of the inhibitory neurons remain ipsilateral while the excitatory interneurons send projections ipsilaterally, contralaterally, and bilaterally (Bannatyne et al., 2009). Physiological study has revealed that both Ia and Ib afferents converge on these interneurons (Czarkowska et al., 1981; Jankowska et al., 1981; Fetz et al., 1979). The distribution of Ib afferents among hindlimb muscles is thought to be greater than that of Ia's (Eccles et al., 1957b; Jami, 1992; Jankowska, 1992).

### **Axotomy Disrupts Stretch Reflex Circuitry**

In order to understand the limitations of the functional recovery of stretch reflex circuitry after peripheral regeneration, one must first recognize the changes induced by the initial injury. This is because restoration of functionality of these circuits necessarily requires that many of these changes be reversed by regeneration. The most obvious change is that transection of a peripheral nerve, also known as axotomy, causes a physical interruption of the connections between mechanoreceptors, afferents, motoneurons, and muscle. Some neurons die as a direct result of injury. Those that do survive undergo a myriad of changes, many of which are reversed upon target reinnervation. Within 24 hours, both sensory and motor neurons begin to undergo Wallerian degeneration distal to the injury site (Griffin et al., 1995). Proximally, cell bodies of injured neurons swell and show signs of chromatolysis in the nucleus (Kreutzberg, 1995). Axon diameters in the proximal stump also decrease, with related decreases in conduction velocity (Czeh et al., 1977; Kuno et al., 1974a). Muscles become paralyzed and undergo denervation atrophy, with muscle mass decreasing by 30% after one week and reaching a plateau by 3 months. This involves both a reduction in muscle fiber caliber and an increase in connective tissue (Sunderland, 1978). Ia afferents, motoneurons, and the synapse between them undergo further cellular and functional changes and are described below.

#### ***Afferent Response to Axotomy***

Afferent cell death occurs in varying degrees depending on the injury model. For example, between 10-30% of sensory neurons die after injury to the sciatic nerve (Arvidsson et al., 1986; Ygge, 1989), although up to 50% of sympathetic neurons in the superior cervical ganglion die after transection of postganglion nerves in as few as 3 days (Hou et al., 1998). Axotomized afferents also show reduced firing of action potentials, with approximately 80%

not firing at all and the remaining afferents firing spontaneously at low rates (Michaelis et al., 2000). Evidence suggests that synapses made by afferents are disrupted after axotomy.

VGLUT1 is the protein responsible for loading glutamate in presynaptic vesicles (Bellocchio et al., 2000, Takamori et al., 2000), and is associated with primary muscle afferents in lamina IX (Alvarez et al., 2004; Oliviera et al., 2003; Todd et al., 2003). It was recently shown that synaptic varicosities of physiologically identified normal Ia afferents always contain high amounts of VGLUT1 (Alvarez et al., 2011), confirming that VGLUT1 is indeed associated with Ia afferents. In sharp contrast to this normally high level, decreases in the expression of VGLUT1 after axotomy suggest that the number of synapses Ia afferents make in the spinal cord is dramatically decreased (Alvarez et al., 2011; Brumovsky et al., 2007; Hughes et al., 2004). Hughes et al. (2004) reported that the luminescence level of VGLUT1 contacts on motoneurons in lamina IX was decreased by 35% after sciatic nerve axotomy when compared to the control side. This is supported by the observation that the number of VGLUT1 contacts on motoneuron cell bodies decreased by half within one week after tibial nerve ligation (Alvarez et al., 2011). The magnitude of all of these findings were shown to decrease with time.

### ***Motoneuron Response to Axotomy***

In sharp contrast to sensory afferents, motoneuron cell loss is estimated at being only between 0–10% as a direct result of sciatic nerve injury (Vanden-Noven et al., 1993; Lowrie et al., 1994; Valero-Cabre et al., 2001). If motoneurons are not allowed to regenerate, however, almost half of them will die (Tornqvist and Aldskogius, 1994). Whole cell intrinsic properties of motoneurons change with the net effect being that they become hyperexcitable. Rheobase current decreases while input resistance and afterhyperpolarization potentials (AHP) increase. These changes have been observed numerous times in both rats (Bichler et

al., 2007; Gardiner and Seburn, 1997; Nakanishi et al., 2005; Yamuy et al., 1992) and cats (Kuno et al., 1974a; Foehring et al., 1986; Gustafsson and Pinter, 1984; Mendell et al., 1995). There is also evidence that different motoneuron types show differences in their response as AHP duration seems to decrease in slow-type motoneurons but increases in fast-type motoneurons (Kuno et al., 1974a). Many of these changes in intrinsic properties may be related to the lack of ACh receptor activation (Nakanishi et al., 2005). It is important to note that the intrinsic properties of uninjured synergist motoneurons remain unchanged after axotomy (Mendell et al., 1995), suggesting that these changes are limited to injured motoneurons. In contrast, uninjured synergist motoneurons show greater than normal activity expressed as an increase in EMG in the uninjured synergist during treadmill walking in cats (Frigon and Rossignol, 2007, 2008; Gritsenko et al., 2001; Pearson et al., 1999). This increased motoneuron activity may suggest an adaptive strategy for overcoming the functional loss of denervated muscle.

After axotomy, motoneurons lose synapses on the soma and proximal dendrites, a process first described by Blinzinger and Kreutzberg (1968) known as synaptic stripping. For example, in the cat hindlimb, the number of synaptic boutons on MG motoneurons was decreased by 83% on the soma and by 57% on the dendrites with insignificant changes on the distal dendrites 12 weeks after transection of the MG nerve (Brannstrom and Kellerth, 1998). There also appears to be a redistribution of synaptic contacts, with S-type (excitatory, glutamatergic) boutons seemingly more affected than F-type (inhibitory, both glycinergic and GABA-ergic) boutons in the rat (Linda et al., 2000). Many of these synaptic contacts return after motoneurons reinnervate muscle, and there seems to be better recovery for S-type boutons than for F-type (Brannstrom and Kellerth, 1999). These findings suggest differential susceptibility to injury and recovery depending on synapse type. The process of synaptic

stripping appears to be controlled at least in part through an immunologic response mediated by major histocompatibility complex (MHC) molecules (Thams et al., 2008).

### *Synaptic Efficacy After Axotomy*

After axotomy, there are observed changes in EPSP amplitude which appear to be time-sensitive. Within three days of axotomy, EPSPs elicited by axotomized Ia afferents are increased in amplitude (Miyata and Yasuda, 1988; Seburn and Cope, 1998). This effect seems to be dependent on Ia afferent injury as stimulation of the axotomized MG nerve elicited greater amplitude EPSPs in either axotomized MG or injury-spared LGS motoneurons, while stimulation of injury-spared LGS afferents elicits EPSPs of normal amplitude regardless of the injury state of the motoneuron (Seburn and Cope, 1998). EPSP amplitude then begins to decline 1-2 weeks after injury (Eccles et al., 1959; Gallego et al., 1979) which suggests a weakening of the Ia-motoneuron synapse. This decline was also observed by stimulation of individual Ia afferents, as the ability generate large EPSP amplitudes was lost (Mendell et al., 1976). Furthermore, connectivity between Ia afferents and homonymous motoneurons, as demonstrated by the presence of an EPSP generated by a single Ia afferent, declined to an average 59% at 60 days or more post-axotomy. In controls, the average connectivity was 96%.

Changes in heteronymous EPSP size have also been observed one week after axotomy and persisting up to at least 3 weeks (Gallego et al., 1979). Different combinations of EPSPs were elicited in triceps surae motoneurons after transection of the MG nerve. Stimulation of axotomized MG nerve elicited monosynaptic EPSPs with decreased amplitude in uninjured LG and SOL motoneurons. However, stimulation of LG or SOL afferents elicited EPSPs with normal amplitudes either in uninjured motoneurons or axotomized MG motoneurons.

This further suggests that changes in EPSP amplitude are restricted to synapses in which Ia afferents had been axotomized. The status of IPSPs generated by axotomized Ia afferents in antagonist motoneurons remains unknown.

### **Regeneration Restores Many Deficits Induced by Axotomy**

Clearly, restoration of function lost after peripheral axotomy requires sensory and motor axons to regenerate and restore connections with their appropriate targets. Successful regeneration and reinnervation depends on a number of factors (Brushart, 2011; Navarro et al., 2007). The age of the subject at the time of injury is the most important factor influencing peripheral nerve injury outcomes. The nerve affected also plays a significant role, with outcomes of smaller, more selective nerves having greater functional recovery than larger, mixed nerves projecting to many muscles. Additionally, the more distal the injury site, the better the outcome. The distance over which the nerve must grow, the time at which surgical repair takes place after injury, and the type of surgical repair used all influence the functional outcome of peripheral nerve regeneration. Even if these factors are minimized, however, the functional recovery remains incomplete (Bisby, 1995). Neurons may die during the regeneration process, and others may innervate inappropriate targets. Damage to tissue and interruption of blood supply may also impede reinnervation. Despite these additional factors, it is clear that recovery of the stretch reflex components, both motor and sensory, is substantial after peripheral nerve regeneration.

There is abundant evidence that the sensory arc of the stretch reflex circuit exhibits good recovery after reinnervation. Over 75% of muscle spindles receive innervation, and over half of these reform annulospiral endings in the equatorial region where Ia afferents endings are normally located (Haftel et al., 2005). Recordings in regenerated dorsal root axons



demonstrate that these afferents do in fact respond to muscle stretch (Banks and Barker, 1989; Lewin and McMahon, 1991). Field potential recordings in the appropriate motor pool reveal that over 40% of Ia afferents respond as if they are connected with muscle spindles in the periphery (Collins et al., 1986). Furthermore, stretch sensitive afferents recover their ability to encode changes in both muscle length and velocity (Haftel et al., 2005). Although muscle afferents can innervate inappropriate targets, these afferents tend to take on the firing characteristics of the end organ they innervate (Banks and Barker, 1989). For example, after cut and surgical repair of the common peroneal nerve, Ib afferents can innervate secondary spindle endings and fire in response to muscle stretch like a normal group II afferent. Therefore one cannot assume that a regenerated afferent exhibiting stretch response profiles similar to Ia afferents were necessarily Ia afferents prior to injury, only that they are Ia-like. Regardless, it is clear that afferents reinnervate muscle spindles, and these afferents are capable of transmitting accurate information about muscle length to the CNS.

Motoneuron recovery after peripheral nerve regeneration is also highly successful. With successful reconnection with muscle, denervation atrophy reverses and muscle weights increase to normal values (Cope and Clark, 1993; Cope et al., 1994). Both motor unit and whole muscle tensions recover (Foehring et al., 1986a; Gordon and Stein, 1982). Changes in the intrinsic whole cell electrical properties, e.g. rheobase, input resistance, and AHP, return to pre-axotomy levels once regeneration has occurred (Bichler et al., 1997; Bullinger et al., 2011a; Foehring et al., 1986a; Mendell et al., 1995). These changes directly depend on the motoneuron successfully reinnervating muscle fibers as these properties do not recover in motoneurons that do not reconnect with muscle fibers (Foehring et al., 1986b; Pinter and Vanden Noven, 1989).

The time course of motoneuron property recovery, along with the time course of the development of muscle tension, can be used to infer the time-course of reinnervation in the cat (Gordon and Stein, 1982; Foehring et al., 1986a, 1986b). After a muscle nerve is cut and surgically rejoined, motor unit tension starts to reappear about one month after injury. By 9-10 weeks, intrinsic motoneuron properties and muscle contractile properties began to change in the direction of normal values. At this time, motor unit types became recognizable, and the proportions of fast and slow motor units present were similar to normal. While recovery of motoneuron properties is still incomplete at 6 months after reinnervation (Mendell et al., 1995), by 9-11 months these properties have returned to normal levels (Foehring et al., 1986a, b). At this time, the maximum tetanic tension of muscle units recovered to normal values, with the exception of those elicited in fast-fatiguing units (Foehring et al., 1986a). It should be noted that Gordon and Stein (1982) considered long-term reinnervation from 5-18 months after injury, and so data taken from this group is hard to interpret. Inclusion of earlier time points before 9 months may explain why whole muscle tension in this group only recovered to 72% of preoperative levels.

Additional observations suggest that synapses on motoneurons return after regeneration. Motoneurons are capable of responding to uninjured sources of excitatory input, such as cutaneous or crossed extension stimulation, which suggests that at least some synapses made with motoneurons become functional (Cope and Clark, 1993; Cope et al, 1994). The efficacy of motoneuron synapses is further supported by observations that electrical stimulation of Ia afferents produces homonymous EPSPs with normal amplitude in regenerated motoneurons at both low frequencies (Bullinger et al., 2011a; Haftel et al., 2005) and in response to high frequency stimulus trains (Bullinger et al., 2011a). Heteronymous connections made by regenerated afferents onto uninjured synergist motoneurons produce decreased EPSP

amplitudes (Eccles et al., 1962; (Mendell et al., 1995). However, separate study by Goldring et al. (1980) observed that EPSP amplitudes returned to normal in synergist motor pools which are predominantly fast-type, even when reinnervation was delayed by 2-6 months. This discrepancy may be accounted for by the observations that Eccles et al. used kittens, and Mendell et al. studied EPSP amplitudes 6 months after the nerve was cut and surgically rejoined, a time when reinnervation is still incomplete (Foehring et al., 1986b). Heteronymous connections made by uninjured afferents onto regenerated motoneurons produce EPSPs with amplitudes that are either normal (Eccles et al., 1962) or increased (Mendell et al., 1995). These observations, taken with those stated above, suggest that the stretch reflex circuit components are intact after peripheral nerve regeneration.

### **Regeneration Fails to Restore Homonymous Stretch Reflexes**

Although both motor and sensory neurons recover to some extent after peripheral nerve injury (Sunderland 1978), restoration of function is incomplete. It has been repeatedly observed that a reinnervated muscle fails to produce homonymous stretch reflexes in both cats (Cope and Clark, 1993; Cope et al., 1994; Huyghues-Despointes et al., 2003; Maas et al., 2007) and rats (Haftel et al., 2005). Although the structural and relative functional recovery of the individual components of the stretch reflex is not 100%, the amount of success achieved as described above suggests there is another player involved in the complete loss of the stretch reflex. Therefore while peripheral factors may contribute to areflexia, growing evidence suggests there is a strong central component that limits the functional recovery of stretch reflex circuitry.

Recent breakthroughs in the study of the cause of areflexia from our lab and others suggests that there is a central disconnect between Ia afferents and motoneurons. Alvarez et al. (2011)

suggests that stretch areflexia is due to the retraction of Ia afferents from motor pools located in lamina IX of the spinal cord. Analysis of the expression of vesicular glutamate transporter VGLUT1, a marker for Ia synapses on motoneurons (Alvarez et al., 2004) was performed after regeneration either the tibial or MG nerve. After nerve regeneration, the synapses made by regenerated Ia afferents were reduced by at least 75% on the soma and by approximately 50% on the proximal dendrites of regenerated motoneurons. This loss was indistinguishable from the effects seen after nerve ligation, suggesting the loss was independent of reinnervation. The retention of other types of synapses on the motoneuron through use of various immunological markers (e.g. with excitatory INs, inhibitory INs, spinal cholinergic INs) suggests synaptic loss is restricted to those made with Ia afferents. To gain additional insight as to the cause of VGLUT1 loss, afferents physiologically identified as exhibiting Ia-like characteristics after regeneration were intra-axonally labeled and their central projections were examined. Further analysis revealed a retraction of Ia afferents from lamina IX after peripheral nerve regeneration. This would suggest that regenerated Ia afferents retract from all motoneurons in lamina IX, but the functionality of these heteronymous synapses remains unknown. A striking observation was that some VGLUT1 synapses in lamina V and VII were retained, although these synapses were significantly reduced in size by about 40% when compared to controls. The functionality of these synapses also remains unknown.

In the companion study from our lab, support for the observed anatomical retraction was generated by establishing the functionality of homonymous synapses made between regenerated Ia afferents and motoneurons in anesthetized rats (Bullinger et al., 2011a). The synaptic response to stretch was found to be impaired, confirming and extending findings made by our laboratory previously using a decerebrate preparation (Haftel et al., 2005). Only 60% of regenerated MG motoneurons produced eSSPs in response to either ramp hold

release stretch or quick, triangular (tendon tap-like) stretch of reinnervated MG muscle. This was in stark contrast to controls, in which muscle stretch produced eSSPs in 100% of motoneurons. In cases where eSSPs were generated after nerve regeneration, the amplitude was reduced by half. If this eSSP reduction is related to the loss of VGLUT1 presented above, it stands to reason there should be an impairment in the ability of individual Ia afferents to elicit excitation in motoneurons. Therefore individual regenerated Ia-motoneuron synapses were studied using the spike-triggered averaging technique which allows for the study of EPSPs in motoneurons generated by single Ia afferents (Mendell, 1971). We found that 80% of individual regenerated MG afferents that do respond to stretch fail to generate EPSPs in regenerated homonymous motoneurons. This again was in stark contrast to controls, where stimulation of physiologically characterized Ia afferents elicited EPSPs in virtually all homonymous motoneurons. In sum, the physiological findings presented above support the role of a central disconnect between Ia afferents and motoneurons after peripheral nerve regeneration.

The functional consequences of lost stretch feedback that still persist after peripheral nerve regeneration are readily appreciated in experiments which observed the locomotor patterns in cats whose triceps surae had been reinnervated by their cut nerves. Since stretch reflexes are lost after reinnervation (Cope and Clark, 1993; Cope et al, 1994), these preparations are lacking a substantial amount of feedback from ankle extensors. Interjoint coordination appears to be disrupted (Abelew et al, 2000; Chang et al., 2009; Maas et al., 2007). Additionally, a significant amount of ankle yield in the treated leg is observed during downslope walking, a condition under which triceps surae muscles experience a greater amount of stretch (Abelew et al, 2000; Maas et al., 2007).

## **CHAPTER 2: General Methods**

### ***Animals***

Experiments were performed using either adult cats (Chapter 3) or adult female Wistar rats (Chapter 4) in accordance with both the Wright State University Laboratory Animals Care and Use Committee and the National Institutes of Health. The switch to the rat model for the studies described in Chapter 4 was made in order to increase the number of experiments that could be performed as well as to decrease the time needed to allow for reinnervation to occur. Food and water were available *ad libitum*, and toys (cats) or chew sticks (rats) were given to encourage activity. Animals were randomly placed into either control or treated groups. The treated groups were subjected to a chronic nerve treatment applied in a survival surgery described below. Animals in the treated group were studied at various time points after survival surgery which were specific to each project. All animals were studied during single terminal experiments for data collection.

### ***Survival surgery***

In order to study changes in the function of neural circuitry underlying stretch-evoked reflexes after reinnervation, we performed surgery on nerves supplying muscles selective to each project. Surgeries were performed using sterile surgical techniques while the animals were anesthetized by inhaled isoflurane. Anesthesia was induced in a species-specific manner. Deep anesthesia was maintained throughout the surgical procedure with inhaled isoflurane (1.0-2.5% in 100% oxygen). Vital signs were monitored during cat survival surgeries given the length of the procedure. A skin incision was made over the popliteal

fossa of the left hindlimb to gain access to the nerves under study. The individual nerves supplying muscles specific to each project were then isolated using blunt dissection under a dissection microscope. Once isolated, the nerves were cut and immediately rejoined end to end through the epineurium with one to two sutures using 10-0 ethilon. The wound was irrigated with 0.9 % saline and individual fat, fascial and skin layers were sutured closed. A subcutaneous injection of buprenorphine (NSAID, species-specific doses) was given before anesthesia was discontinued. Animals were returned to their cages upon discontinuation of anesthesia and were monitored throughout the recovery period. Additional NSAIDs were given for the next 48 hours and animals were monitored for signs of stress or infection. All animals received regularly scheduled veterinary care until the time of terminal surgery.

### ***Terminal experiment***

#### ***Anesthesia***

Anesthesia was induced and initially maintained in the same manner as survival surgeries. All animals were intubated for terminal experiments in order to maintain a patent airway and the carotid arteries were ligated bilaterally. In order to ensure that deep anesthesia was maintained throughout the terminal experiment, heart rate, blood O<sub>2</sub> saturation, expired CO<sub>2</sub>, temperature, respiratory rate, and in some instances blood pressure (cat) were monitored. These vital signs were maintained according to species-specific parameters through the adjustment of isoflurane concentration, adjustment of radiant and water-pad heat sources, and administration of Ringer-dextrose solution. Withdrawal reflexes were also monitored to ensure they remained suppressed.

### *Surgical Preparation*

The surgical preparation was the same for all terminal studies. After anesthesia was induced, muscles and nerves specific to each project were isolated either ipsilaterally (rat) or bilaterally (cat). Resting lengths of each muscle corresponding to 90° flexion of the knee and ankle were marked with suture. Tendons were cut while retaining a piece of the calcaneus at the end of each tendon. The caudal cutaneous sural nerve and the posterior tibial nerve were isolated bilaterally and cut to allow for stimulation. Plastic nerve cuffs fitted with bipolar electrodes were secured around each posterior tibial nerve for stimulation later in the experiment (see below). The animal was then moved to a stereotaxic frame where the head, spine and hips were secured. Hindlimbs were secured at the knee and ankle (approximately 135° and 90°, respectively). Pairs of fine-wire electrodes were inserted into the muscles under direct study for electromyographic (EMG) recording. Since reflex contraction of muscle is suppressed by isoflurane, the animal was rendered insensate by performing a decerebration. This was accomplished by removing all brain tissue rostral to a mid-collicular transection. Gaseous anesthesia could then be discontinued in order to study whole muscle response to stretch. All vital sign monitoring continued until the end of the experiment when the animal was euthanized.

### *Muscle Reflex Assessment*

Both homonymous and heteronymous stretch-evoked muscle reflexes were assessed by tying muscle tendons to force transducers in parallel with length-servo motors controlled by customized software. Muscle force, muscle length, and EMG were recorded, digitized (20 kHz) and stored on a computer for later analysis using CED Spike 2 software. Reflexes were evoked by ramp-hold-release stretches following the paradigms detailed in each project. Stretches were applied when the muscle was inactive (quiescent state) and held at resting

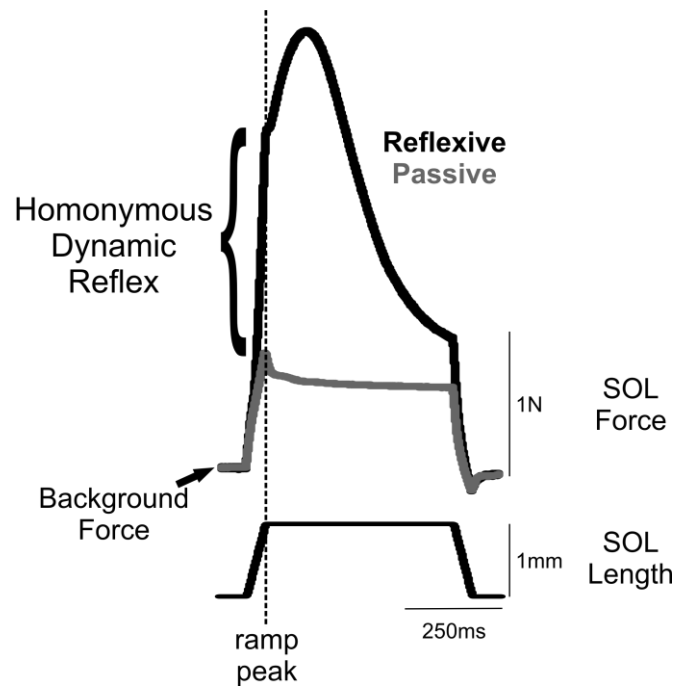


length, or during active reflex contraction (active state) elicited by electrical stimulation (100Hz) of either the ipsilateral caudal cutaneous nerve or the contralateral posterior tibial nerve through the crossed extensor reflex. Since force responses to stretch contain both a intrinsic component due to the non-reflexive mechanical properties of muscle in addition to the reflexive component (Nichols and Houk, 1976), the passive intrinsic component was estimated at the end of each experiment by recording the force response to stretch of the muscles after the nerve supplying each muscle was cut. In order to assess the force-generating capacity of the muscle, the maximum tetanic tension was obtained after passive response collection by recording the maximum force response to electrical stimulation (50Hz) of the cut nerve supplying select muscles under study while the muscle was held at resting length.

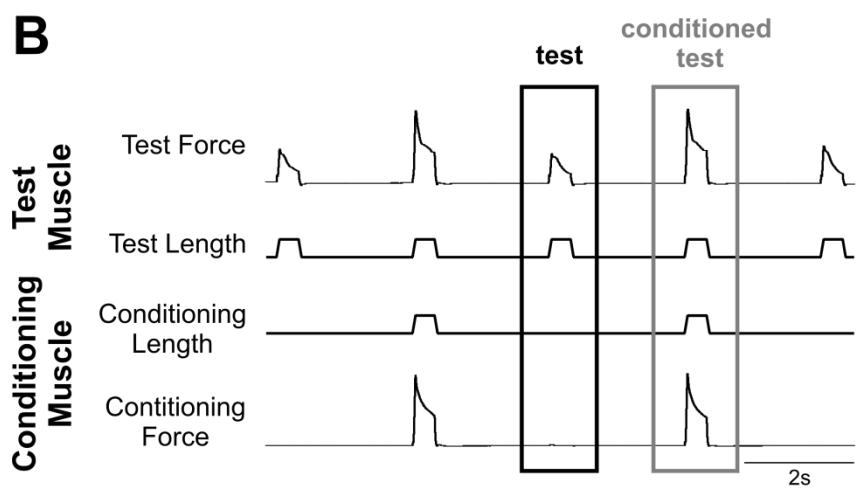
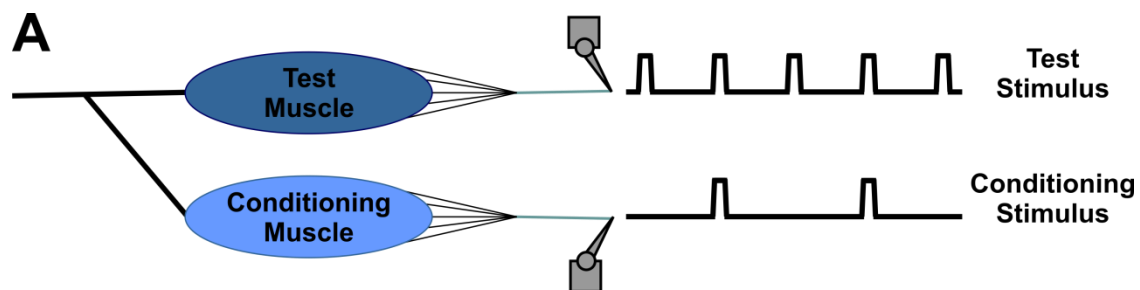
Homonymous stretch-evoked reflexes were assessed by stretching a single muscle alone. Forces were measured at the peak of the ramp stretch (dynamic force). Quantification of the force responses was calculated subtracting the averaged force occurring over the 15s prior to the onset of the ramp (background force) from the dynamic force. The force directly attributable to reflex was calculated by subtraction of the passive response and is referred to as the homonymous dynamic reflex (Figure 2.1).

Since stretch of a muscle alone may not be sufficient to produce a heteronymous force response in a second muscle, heteronymous stretch-evoked reflexes were assessed by stretching muscles in pairs. The basic protocol is shown in Figure 2.2A. One muscle was stretched on each trial and is termed the test stimulus ("test muscle" traces in Figure 2.2B). Force responses to stretch of this muscle alone (test, black box) represent the test response

**Figure 2.1** *Homonymous stretch reflex assessment.* Dynamic forces were recorded at the peak of ramp stretch. The passive intrinsic response (mechanical response, nerve cut; gray trace) was then subtracted from the dynamic force to calculate the dynamic force due to reflex alone (homonymous dynamic reflex, black trace).



**Figure 2.2 *Heteronymous stretch reflex assessment.*** A: Schematic of the stretch paradigm. One muscle is stretched on every trial and is called the test stimulus (test muscle). The other muscle is stretched on every other trial and is called the conditioning stimulus (conditioning muscle). B: Isometric force and length of the test muscle (test stimulus) and conditioning muscle (conditioning stimulus) in untreated cat leg during quiescence. The black box is the test response (test muscle stretched alone), while the gray box is the conditioned response (test muscle stretched together with conditioning muscle). C: Heteronymous contributions were calculated by subtracting the test response from the conditioned test response. The resulting force difference at the peak of ramp stretch is referred to as heteronymous dynamic reflex (a). The force occurring immediately before stretch is referred to as the background force (b).



and is comprised of both the homonymous stretch reflex and the intrinsic response. The other muscle was stretched on alternate trials (conditioned test, gray box) and is termed the conditioning stimulus ("conditioning muscle" traces in Figure 2.2B). This muscle will be the source of heteronymous input. Stretching the two together results in a conditioned response of the test muscle (conditioned test), consisting of both homonymous and heteronymous reflex components in addition to the intrinsic response. By using this protocol, heteronymous contributions from the conditioning muscle to the stretch reflex of the test muscle could be estimated. The test response was subtracted from the conditioned-test response, leaving only the heteronymous contribution from the conditioning muscle. These calculations were made for the dynamic force and the resulting force difference is referred to as the heteronymous dynamic reflex (Figure 2.2C). A single trial consists of one sequential pair of test and conditioned test responses.

### *Euthanasia*

All animals were euthanized after undergoing a single terminal experiment. Cats were euthanized by barbiturate overdose (intravenous euthasol, 100 mg/kg). Rats were euthanized by inhaled isoflurane overdose (5% in 100% O<sub>2</sub>) and subsequent exsanguination.

### *Statistics*

In some cases, treatment effects on heteronymous stretch reflexes were assessed by comparing the right (untreated) leg to the left (treated) leg for individual experiments. Student's independent t-test was used to test for statistical significance between legs (Statistica Software package). In other cases, treatment effects were assessed by pooling data and comparing across treated and control groups. Analysis of Variance (ANOVA) was used to test for statistical significance between groups. Tukey's honestly significant difference

(HSD) post hoc test was used to account for differences within groups (SYSTAT, Systat Software, Point Richmond, CA). The non-parametric Fisher's exact test was used to test for significance between percentages. The non-parametric Kolmogorov-Smirnov test was used to test for significance in the distribution of data values. Skewness was calculated for each group to further interpret differences in data set distribution. Data are reported as mean  $\pm$  SEM unless otherwise noted. The level of significance was set at  $p < 0.05$  for all statistical tests.

## **CHAPTER 3: Disruption of stretch feedback among synergists after reinnervation**

### **Introduction**

It is firmly established that proprioceptive feedback is disrupted after regeneration of a transected peripheral nerve as homonymous stretch reflexes are permanently lost (Cope and Clark, 1993; Cope et al., 1994; Huyghes-Despointes et al., 2003; Haftel et al 2005; Maas et al 2007). This is despite evidence that many portions of the stretch reflex circuit recover. Over 75% of muscle spindles receive appropriate innervation (Haftel et al., 2005), 50% of regenerated Ia afferents respond to stretch (Collins et al 1986), and stretch sensitive afferents encode changes in length and velocity as they do normally (Haftel et al., 2005). Regenerated Ia afferents evoke EPSPs of normal amplitude in regenerated motoneurons both at low frequencies (Bullinger et al., 2011a; Haftel et al., 2005) and in response to higher, physiologically relevant frequencies (Bullinger et al., 2011a). Furthermore, regenerated motoneurons are also capable of firing in response to other sources of excitatory input (Cope et al 1994). The failure of muscle stretch to produce homonymous reflex contraction may decrease force production as excitatory drive to motoneurons is substantially reduced after deafferentation (Goldberger and Murray, 1974; Wetzel et al., 1976). It also leads to impaired limb coordination, as after regeneration coordination is impaired in both animals (Abelew et al., 2000, Maas et al., 2007; Chang et al., 2009) and humans (Sainburg et al., 1993; Gordon et al., 1994a-b; Gordon et al 1995).



However, disruption of stretch reflexes may not be limited to homonymous pathways. This is because Ia afferents distribute synaptic input to both themselves as well as the motor pools of synergistic muscles (Eccles et al., 1957; Nichols 1989; Nichols 1999). Recent evidence suggests that areflexia results in part because peripherally-regenerated Ia afferents retract collateral axon branches that normally project into lamina IX motor pools (Alvarez et al., 2011). This regional retraction of axon collaterals would seem to deprive afferent input to all motoneurons, both homonymous and heteronymous, regardless of injury. This is in contrast to the process of synaptic stripping where axotomized motoneurons lose their synapses (Blinzinger and Kreutzberg, 1968; Brannstrom and Kellerth, 1998, 1999; Chen, 1978; Linda et al., 2000; Sumner, 1975, 1976), and the loss of Ia afferent connections would subsequently be restricted to injured motoneurons. Therefore if collateral axon retraction is a regional phenomenon and not selective for damaged motoneurons, then regenerated afferents should fail to generate heteronymous stretch reflexes in multiple synergistic motor pools, even in ones that are injury-spared. The functionality of these regenerated heteronymous Ia projections after peripheral nerve regeneration, both regenerated and injury-spared, remains relatively unknown, although there have been hints they may also be disrupted (Cope et al., 1994). The primary goal of this study was to directly test the functionality of the circuits underlying stretch-evoked reflexes among a group of synergists, one of which was injury-spared.

This experimental design also provides for a unique opportunity to study the capability of spinal circuits to adapt in response to peripheral nerve injury. Proprioceptive input from an injury-spared muscle is in a prime position to compensate for lost force and proprioceptive feedback from reinnervated synergists. Compensation from this source necessarily requires some modification as synaptic excitation is normally much weaker from heteronymous than

from homonymous proprioceptive inputs (Nichols, 1999). Amplification of heteronymous synaptic excitation from the injury-spared muscle would therefore be required in order to make up for lost homonymous proprioceptive excitation long after nerve repair and regeneration. This amplification would also be necessary because early after nerve injury, more recruitment of motoneurons in injury-spared muscle is needed to make up for both lost synergist force and heteronymous feedback. Our secondary goal was to study these possibilities.

The capability of injury-spared proprioceptive inputs to adapt to synergist nerve injury has been the subject of previous study, with differing results. In a series of experiments, the responses of cats to partial paralysis of ankle extensor muscles were studied. The ankle yields immediately, but gradually returns toward normal over several days (Pearson et al., 1999; Gritsenko et al., 2001; Frigon and Rossignol, 2007, 2008), even in spinal cats (Bouyer et al., 2001). In association with partial kinematic recovery, Pearson et al. found that soon after injury, electromyography (EMG) activity in injury spared muscle increased at a time attributable to proprioceptive drive occurring during stance. With time, it appeared that descending drive caught up to the increased contribution made by proprioceptive inputs. In contrast, Gritsenko et al (2001) did not find the differential time course of EMG increase in late versus pre-EMG components. Therefore there is no resolution of possible proprioceptive feedback compensation early after injury, but this resolution is difficult on the basis of EMG alone. Only one previous study directly examined adjustments in proprioception of the injury-spared muscle after synergist muscles were successfully reinnervated (Maas et al., 2007). Adjustments in proprioception in the form of length feedback were measured directly by recording the force response to stretch (stretch reflex). The homonymous SOL stretch reflex was shown to be entirely suppressed, but the state of heteronymous stretch reflexes

was not reported. Present electrophysiological evidence comes to disparate conclusions about the functionality of these heteronymous synapses as well. Studies at the Ia-motoneuron synapse reveal that EPSP amplitude in regenerated motoneurons remained normal when elicited by electrical stimulation of injury-spared nerves (86-99% of control, Eccles et al., 1962). However, study by Mendell et al. (1995) revealed that the EPSP amplitude was increased by 25%. Taken together, these studies do not resolve the issue of whether or not injury-spared proprioceptive inputs are capable of compensation after synergist nerve injury.

The primary objective of the present study was to directly test the functionality of spinal circuits underlying stretch reflexes among a group of synergists, one of which was injury-spared. This was accomplished by directly measuring muscle force output in response to both homonymous and heteronymous muscle stretch. The nerves supplying the medial and lateral gastrocnemius were cut and surgically rejoined, leaving the soleus nerve intact. The status of SOL homonymous stretch reflexes was assessed through measurement at various time points based on the time course of reinnervation (Foehring et al., 1986). Heteronymous stretch reflexes were measured among the injury-spared SOL and individually reinnervated G muscles long after reinnervation had been established. We found that heteronymous stretch reflexes initiated by reinnervated muscle were dramatically decreased in both regenerated and injury-spared synergist motoneuronal pools, confirming and extending the results of earlier study (Cope et al., 1994). Additionally, both homonymous and heteronymous SOL stretch reflexes were reduced after synergist reinnervation. These results give physiological evidence for retraction of regenerated Ia afferents from all synergist motor pools, and this retraction may extend to afferents that are injury-spared. Furthermore, injury-spared muscle

which is in a position to compensate for lost synergist proprioceptive feedback does not do so. Preliminary results have been presented in abstract form (Horstman et al., 2009; Horstman et al., 2011).

## **Methods**

### ***Animals***

Data were obtained from 12 adult cats of either sex weighing between 2.7 kg and 5.0 kg. Cats were separated into two groups, either treated or control. Nine cats underwent a chronic nerve treatment applied in a survival surgery. Three cats were either untreated or sham-treated. All cats were studied during single terminal experiments, after which they were euthanized by barbiturate overdose (euthasol, 100mg/kg). All procedures were approved by both the Wright State University Laboratory Animals Care and Use Committee and the National Institutes of Health.

### ***Survival surgery***

In order to study the functionality of spinal circuits underlying stretch-evoked reflexes among a group of synergists, we performed surgery on nerves supplying select muscles of the triceps surae. The preanesthetics ketamine and xylazine (10mg/kg, 1mg/kg) were given by intramuscular (IM) injection. Once withdrawal, corneal and swallow reflexes were absent, cats were intubated and an intravenous (IV) catheter was inserted in the right cephalic vein for administration of fluid (lactated ringer; 10mL/hr). Deep anesthesia was maintained throughout the surgical procedure with inhaled isoflurane (1.2-2.5% in 100% oxygen). Heart rate, ECG, temperature, expired CO<sub>2</sub> and respiration rate were all recorded to ensure anesthesia was maintained. Using sterile surgical procedures, a 5 cm incision was made longitudinally over the popliteal fossa in the left hindlimb. Tissues were retracted to allow

access to the nerves supplying the MG, LG and SOL muscles. The individual nerves were isolated under a dissection microscope, and individual nerve branches were electrically stimulated with bipolar electrodes (2Hz, pulse width 0.04ms). The identity of the isolated nerve was verified when its parent muscle contracted in response to stimulation. A 1.5 cm incision was made over the distal third of the SOL muscle to allow for confident visualization of SOL contraction. In one cat, branches of the SOL nerve were not separable from the LG nerve during survival surgery so the cat was used as a sham control. In all other cats that underwent survival surgery, the branches supplying the MG and LG were then cut approximately one cm from the muscle. In seven cats, the MG and LG nerves were immediately rejoined through the epineurium with one to two sutures (10-0 ethilon). In two cats, the MG and LG nerves were ligated by tying two pieces of suture (10-0 ethilon) around the nerve 3-4mm apart. 3 mm sections of nerve were excised between the two suture ligatures, with the proximal end subsequently sutured into the popliteal fat pad. Repetition of electrical stimulation of the SOL nerve verified nerve function (see above). Individual fat, fascial and skin layers were sutured closed (4-0 absorbable vicryl) at both incision sites. A subcutaneous injection of buprenorphine (0.3mg/kg, NSAID) and an IM injection of antibiotic were given before discontinuation of anesthesia. Cats were continuously monitored until sternal position was recovered. Additional oral NSAIDs were given for the next 48 hours, and oral antibiotics were continued for the next 5 days. Cats recovered in cages for 5-7 days after the procedure and were monitored for signs of infection or discomfort. They were then released back into open floor housing where they had ample room to run and climb. Cats received regularly scheduled veterinary care until the time of terminal surgery.

## ***Terminal Experiment***

### *Surgical preparation*

The surgical preparation was the same for all terminal studies. Anesthesia was induced and initially maintained in the same manner as survival surgeries. After intubation, IV lines were inserted into both the right jugular vein as well as the right cephalic vein for delivery of fluid and drugs. The carotid arteries were ligated bilaterally, and a cannula connected to a blood pressure transducer was inserted into the right carotid artery proximal to ligation. Heart rate (100-160 bpm), blood pressure (mean 60-120 mmHg), blood O<sub>2</sub> saturation (90-100%), expired CO<sub>2</sub> (35-45%), temperature (35-38°C), respiratory rate (adjusted to maintain CO<sub>2</sub>), and withdrawal reflexes were monitored to ensure that deep anesthesia was maintained. The SOL, MG and LG muscles and nerves were each isolated bilaterally. Resting lengths of each muscle corresponding to 90° flexion of the knee and ankle were marked with suture.

Tendons were cut while retaining a piece of the calcaneus at the end of each tendon. The caudal cutaneous sural nerve and the posterior tibial nerve were isolated bilaterally and cut to allow for stimulation. Plastic nerve cuffs fitted with bipolar electrodes were secured around each posterior tibial nerve for stimulation later in the experiment (see below). The animal was then moved to a stereotaxic frame where the head, spine and hips were secured.

Hindlimbs were secured with clamps at the knee and ankle (approximately 135° and 90°, respectively) attached to the mechanical ground by magnetic bases. Two pairs of fine-wire electrodes were inserted into each muscle for EMG recording. Since reflex contraction of muscle is suppressed by isoflurane, the animal was rendered insensate by performing a decerebration. This was accomplished by removing all brain tissue rostral to a mid-collicular transection. Gaseous anesthesia could then be discontinued in order to study whole muscle response to stretch. All vital sign monitoring continued until the end of the experiment when the cat was euthanized (see above).

### *Muscle Reflex Assessment*

The functionality of projections of regenerated Ia afferents to heteronymous synergist motor pools was determined by recording the whole muscle force response to stretch. Therefore a thorough investigation of both homonymous and heteronymous stretch reflexes was performed among the triceps surae one year after the MG and LG nerves were cut and surgically rejoined (n=3). This is because reinnervation is nearly complete by one year (Gordon and Stein, 1982; Foehring et al., 1986a); before one year, the force responses to stretch in treated muscles may be confounded by the progress of reinnervation. In order to examine the capability of injury-spared SOL homonymous reflexes to adapt to changes in reinnervated G feedback and force, a second set of terminal experiments was performed where homonymous SOL stretch reflexes were studied in relation to the time course of reinnervation. These included a time point before reinnervation begins (3 weeks, n=2) as well as a time when reinnervation was well under way (12 weeks, n=2; Foehring et al., 1986b). In order to study the effects of preventing reinnervation on an injury-spared synergist, the two animals that underwent ligation were also studied at 12 weeks. The sham-treated was studied 3 weeks after survival surgery and was included in this set of experiments along with the other two controls. SOL homonymous stretch reflex data collected in the first set of experiments at the one year time point was included in this analysis.

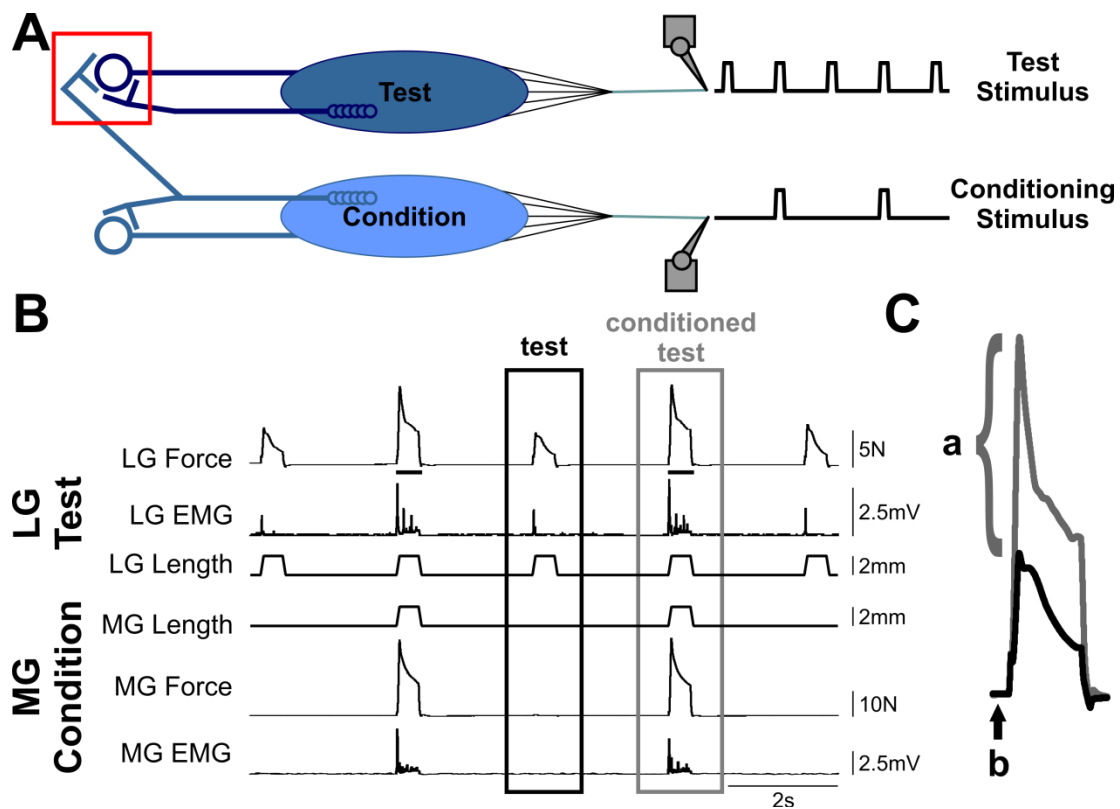
Both homonymous and heteronymous muscle reflexes were assessed as described previously (Huyghues-Despointes et al., 2003; Nichols, 1999). Briefly, muscle tendons were tied to force transducers in parallel with length-servo motors controlled by customized software. Muscle force, muscle length, and EMG were recorded, digitized (20 kHz) and stored on a computer for later analysis using CED Spike 2 software. Reflexes were evoked by ramp-

hold-release stretches following one of two paradigms according to the time after survival surgery the terminal experiment was performed. Experiments at the one year time point ( $n=3$ ) used length perturbations with ramp duration = 50ms, amplitude = 2mm, and hold period = 350ms. The second set of terminal experiments designed for homonymous SOL study at earlier time points ( $n=9$ ) used ramp duration = 50ms, amplitude = 1mm, and hold period = 500ms. Muscles were stretched at 2s intervals in order to decrease history dependence yet still maximize data collection at elevated force levels. Stretches were applied when the muscle was inactive (quiescent state) and held at resting length, or during active reflex contraction (active state) elicited by electrical stimulation (100Hz) of either the ipsilateral caudal cutaneous sural nerve or the contralateral posterior tibial nerve through the crossed extensor reflex. Since force responses to stretch contain both a intrinsic component due to the non-reflexive mechanical properties of muscle in addition to the reflexive component (Nichols and Houk, 1976), the passive intrinsic component was estimated at the end of each experiment by stretching the muscles after the nerve supplying each muscle was cut. In order to assess the force-generating capacity of the SOL in relation to muscle weight, the maximum tetanic tension (tetanic force) was obtained after passive response collection by recording the maximum force response to electrical stimulation (50Hz) of the cut LGS nerve.

Homonymous and heteronymous stretch reflexes were assessed as detailed in Chapter 2 and will be briefly summarized here. Homonymous stretch reflexes were assessed by stretching a single muscle alone where the background force was subtracted from the force measured at the peak of the ramp stretch (dynamic force). The force directly attributable to reflex was calculated by subtraction of the passive response and is referred to as the homonymous dynamic reflex. Since stretch of a muscle alone may not be sufficient to produce a heteronymous force response in a second muscle, heteronymous stretch reflexes were



**Figure 3.1 *Heteronymous stretch reflex assessment between synergists.*** A: Muscle stretch paradigm and underlying neural circuitry. One muscle was stretched on each trial and is termed the test stimulus (muscle labeled "Test") while the other muscle was stretched on alternate trials and is termed the conditioning stimulus (muscle labeled "Condition"). Open forks: excitatory connection from Ia afferent whose axon innervates muscle spindles. Open circles: motoneuron cell body whose axon projects back to muscle. The synapse under study is shown in the red box. B: Isometric force, length, and EMG (rectified and integrated) traces from experiment 3 (E3) of LG (test stimulus) and MG (conditioning stimulus) in the untreated leg during quiescence. The black box is the test response (LG stretched alone), while the gray box is the conditioned response (LG stretched together with MG). Black bars underneath LG force trace indicate conditioned test response. C: Heteronymous contributions were calculated by subtracting the test response from the conditioned test response. The resulting force difference is referred to as heteronymous dynamic force (peak force at the top of the ramp stretch, a). The force occurring immediately before stretch is referred to as the background force (b).



assessed by stretching muscles in pairs. The stretch protocol and the synapse under study is shown in Figure 3.1A with corresponding control data traces shown in Figure 3.1B. One muscle was stretched on each trial and is termed the test stimulus ("LG Test" traces in Figure 3.1B). Force responses to stretch of this muscle alone (test, black box) represent the test response and is comprised of both the homonymous SR and the intrinsic response. The other muscle was stretched on alternate trials and is termed the conditioning stimulus ("MG Condition" traces in Figure 3.1B). This muscle will be the source of heteronymous input. Stretching the two together results in a conditioned response of the test muscle (conditioned test, gray box), consisting of both homonymous and heteronymous reflex components in addition to the intrinsic response. In order to estimate the heteronymous reflex contribution, the test response was subtracted from the conditioned test response, leaving only the heteronymous contribution from the conditioning muscle. These calculations were made for the dynamic force and the resulting force difference is referred to as the heteronymous dynamic reflex (Figure 3.1C, a). A single trial consists of one sequential pair of test and conditioned test responses. Since only two muscles could be connected to motors at any given time, either a 100g weight or a 50g weight was hung from the muscle that was not connected (G or SOL, respectively) to approximate resting force.

### *Nerve Histology*

In order to assess possible damage to the SOL nerve during survival surgery, the SOL nerves from treated cats were analyzed histologically by Dr. Charlotte Sumner. Briefly, a 1.5 cm portion of the SOL nerve from the area in which the LG nerve branches were cut and surgically rejoined was transected and fixed with 4% gluteraldehyde overnight, then switched to 0.1M Sorenson's phosphate buffer. Nerve sections were put on ice and shipped overnight for processing and analysis. Nerves were cut into 1 $\mu$ m sections, stained with Toluidine blue

and examined under a light microscope for aberrations in axon and myelin sheath morphology.

### *Statistics*

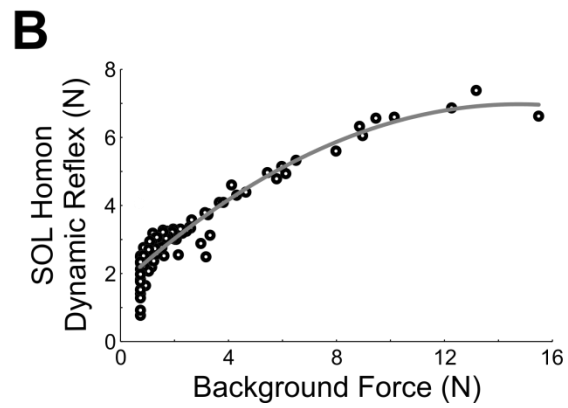
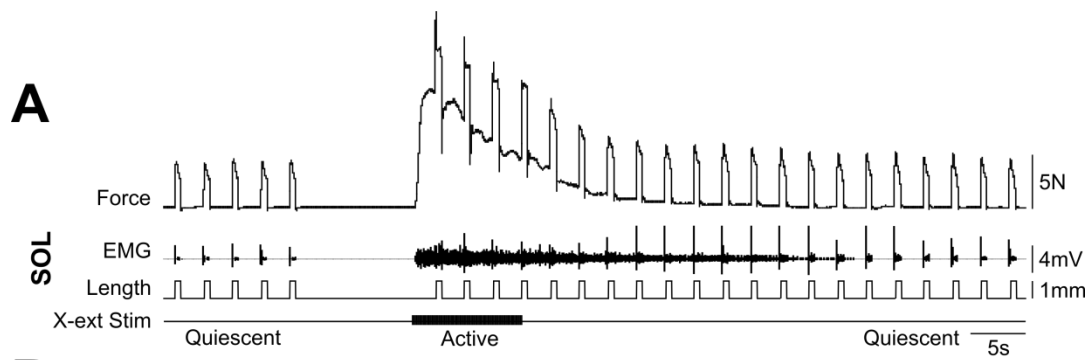
Treatment effects on heteronymous stretch reflexes were assessed by comparing the right (untreated) leg to the left (treated) leg for individual experiments. Student's independent t-test was used to test for statistical significance between legs (Statsitica Software package). Treatment effects on homonymous stretch reflexes were assessed by comparisons between control and treated animals. Data are reported as mean  $\pm$  SEM unless otherwise noted.

## **Results**

### *Factors that influence stretch reflex amplitude*

There are a number of factors that can affect reflex force in control animals. Animal weight may have an influence on load bearing; increases in weight born by the animal could increase the force of muscle contraction, thereby affecting reflex force amplitude. Furthermore, stretch reflex amplitude decreases with age as motor unit firing rate is impaired (Erim et al., 1999; Soderberg et al., 1991). These factors were controlled for at the outset by only using animals aged 1-2 years old with weights from 2.7-5.0 kg. The amount of background force also influences the amount of reflex amplitude as larger motor units are progressively recruited (Matthews, 1968). Therefore comparisons of stretch reflex amplitude were made at matched background forces. The amount of background force was modulated in these experiments by stimulation of either the contralateral posterior tibial nerve (crossed extension reflex) or the ipsilateral caudal cutaneous sural nerve (sural reflex). Typical SOL force responses to ramp hold release stretch in a control cat under both quiescent and active conditions are shown in Figure 3.2A. After the initial quiescent collection phase, a crossed

**Figure 3.2 *Factors affecting stretch reflex amplitude.*** Amount of reflex force is correlated with background force (A-B). A: Raw force, length and EMG traces for SOL response to ramp hold release stretch in a control cat. Quiescent data before and after crossed extension were used for quantitative analysis while active data was used for qualitative analysis only. B: Plot of homonymous dynamic reflex force (Homon Dynamic Reflex) versus background force for the same cat during both quiescence and crossed extension. It should be noted that a small portion of this reflex force is due to the increased active intrinsic response. Data points were fit with a polynomial curve ( $r=0.9154$ ,  $p<0.001$ ).



extension reflex was elicited (active phase). Once the stimulus was turned off, background force returned to pre-crossed extension levels and background EMG activity ceased. At this point, the preparation was considered to have returned to a quiescent state. The increase in homonymous stretch reflex amplitude with increased background force is readily appreciated in Figure 3.2B. There is a clear correlation between reflex force and background force ( $r=0.9154$ ,  $p<0.001$ ), although it should be noted that a small portion of this force is due to increases in the active intrinsic component (e.g. Nichols and Houk, 1976). During crossed extension or sural reflexes, there was a great deal of variability and often the best-fit polynomial did not accurately represent the data points. Furthermore, during heteronymous reflex assessment, it was often difficult to obtain test -conditioned test pairs at matched background forces. As such, quantitative comparison was often difficult and unreliable at higher background forces. Therefore quantitative analysis of both homonymous and heteronymous stretch reflexes was made during the quiescent state at resting length, while reflexes obtained during the active state were used for qualitative analysis only. With these factors that influence stretch reflex amplitude controlled, we now turn to study of stretch reflexes in animals in which the MG and LG nerves have been cut and surgically rejoined.

#### *Regenerated afferents are ineffective in initiating heteronymous SRs*

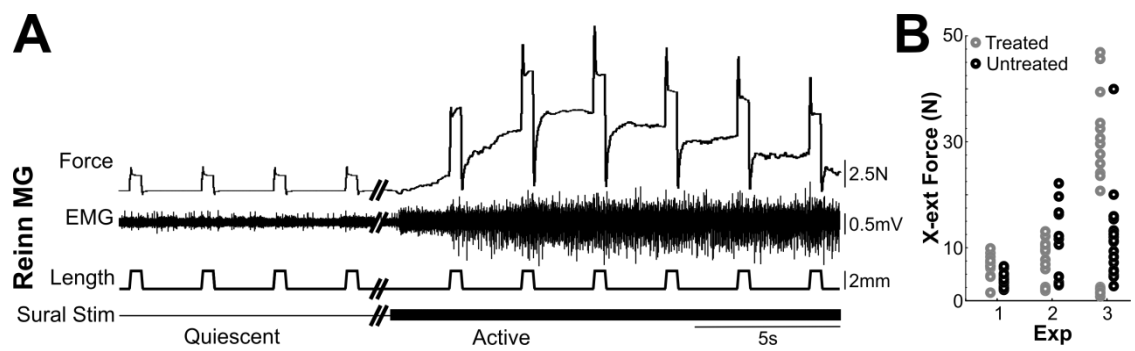
One observation that is critical to the success of determining the functional status of heteronymous projections of regenerated Ia afferents is that reinnervated muscles produced little if any reflex force in response to ramp hold release stretch, in accordance with previous studies (Cope and Clark, 1994; Cope et al., 1994; Hughueyes-Despointe et al., 2003; Haftel et al 2005; Maas et al., 2007). This held true even during conditions where motor pool

excitability was elevated by crossed extension reflexes or ipsilateral sural stimulation (Figure 3.3A). Although it appears that there is an increased force response during the active state, this increase is due solely to an increase in the active intrinsic response (Nichols and Houk, 1976; Huyghes-Despointes et al., 2003a). This was confirmed by the observation that in almost all cases stretch of the reinnervated muscle produced no modulation of EMG activity. In only one case was the presence of increased EMG activity observed, but the force produced by this reinnervated MG muscle was less than 1% of the untreated leg (untreated homonymous reflex:  $19.3917 \pm 0.2525\text{N}$ ,  $n=34$ ; treated homonymous reflex:  $0.2568 \pm 0.02950\text{N}$ ,  $n=48$ ). The lack of a homonymous stretch reflex in reinnervated muscle occurs despite the observation that reinnervated muscles responded to these uninjured sources of excitatory input and achieved force levels comparable to the contralateral side (Figure 3.3B). Furthermore, there was good indication of successful reinnervation of muscle fibers by motoneurons as muscle weights were within 16% of the untreated side (Table 3.1).

With confirmation of the failure of reinnervated muscle to produce homonymous stretch reflexes, we can now turn to an examination of the functionality of synapses made by regenerated afferents on synergist motor pools. The observation that regenerated Ia afferents retract from lamina IX (Alvarez et al., 2011) would suggest that these afferents retract from both homonymous and heteronymous synergist motor pools. The loss of homonymous stretch reflexes taken together with both reductions in SSP amplitude (Bullinger et al., 2011a; Haftel et al., 2005) and a loss of connectivity between single Ia afferents and motoneurons (Bullinger et al., 2011a) clearly indicate that the functionality of synapses between regenerated Ia afferents and regenerated homonymous motoneurons is reduced. However, the functionality of synapses between regenerated Ia afferents and regenerated heteronymous synergist motoneurons remains unknown. This was directly tested by recording the



**Figure 3.3** *Reinnervated muscle fails to initiate homonymous stretch reflexes.* A: Isometric force, length and EMG traces for reinnervated (Reinn) MG homonymous test responses to ramp hold release stretch. Data were collected with the muscle not contracting (quiescent) as well as when the muscle was engaged in ongoing contraction (active state) elicited by sural nerve reflexes. Note the absence of additional EMG activity associated with muscle stretch. B: The maximum background force produced by either crossed extension or sural nerve reflexes (X-ext) in the G muscles for all three reinnervated cats in both the untreated (black circles) and treated (gray circles) legs.



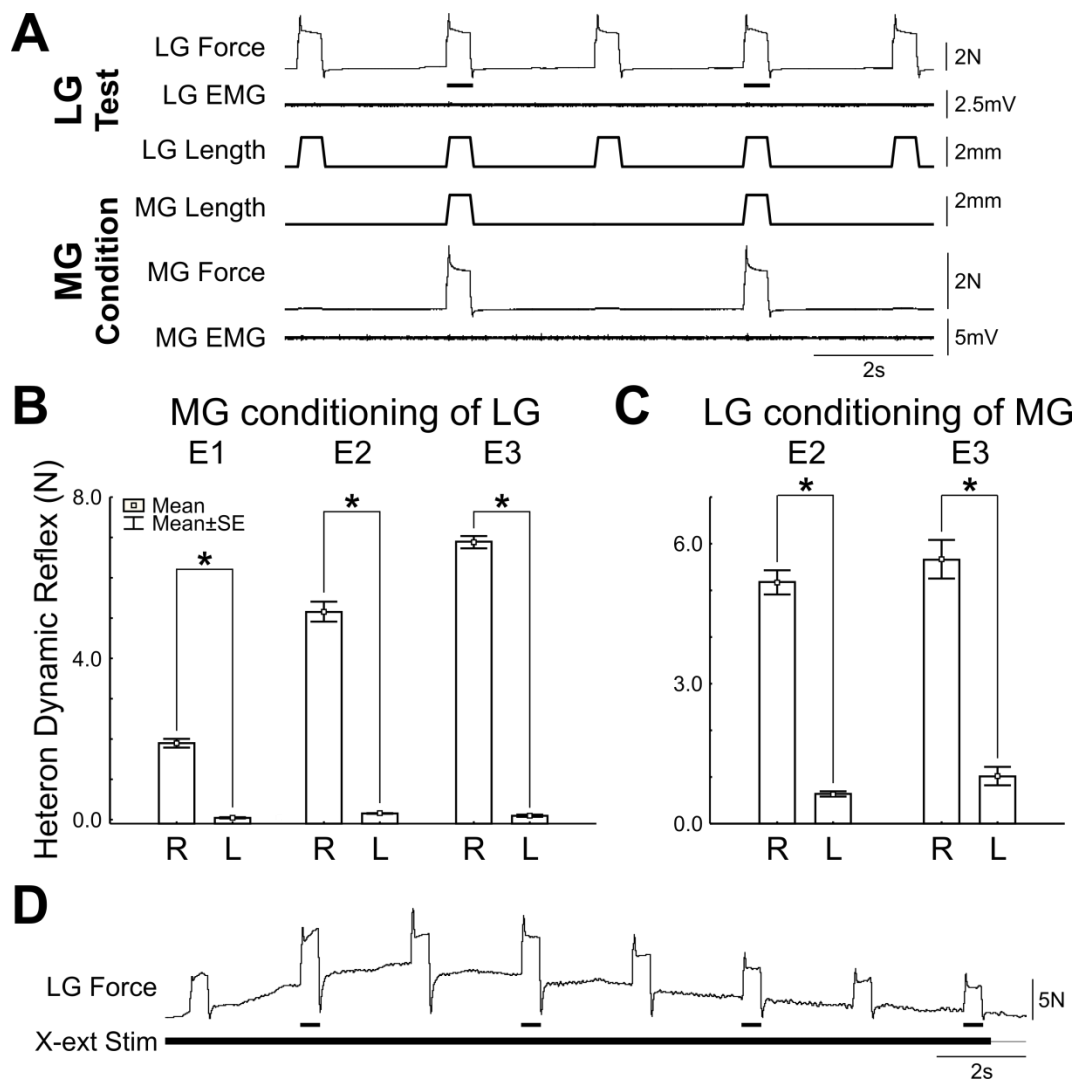
**Table 3.1 *Muscle weights at various stages of reinnervation.*** Wet muscle weights for all triceps surae muscles were obtained immediately after each experiment was completed. Muscle weights from the treated leg (left leg) were expressed as a percentage of the untreated leg (right leg) for all treatment groups in order to estimate the amount of reinnervation that had occurred or, in the case of ligation, had been prevented. Reinn: reinnervation. %Reinn: total G weight from treated leg expressed as a percentage of G weight in untreated leg. %SOL: SOL weight from treated leg expressed as a percentage of SOL weight in untreated leg. E1, E2, and E3 denote experiment numbers for animals studied one year after G nerve was cut and rejoined.

Treatment	Right Leg Muscle Weight (g)				Left Leg Muscle Weight (g)				%Reinn	%SOL
	SOL	MG	LG	G total	SOL	MG	LG	G total		
Control	3.36	11.64	12.99	24.63	3.52	12.05	12.7	24.75	100	105
Control	3.21	9.66	10.06	19.72	3.23	10.19	10.6	20.79	105	101
Control (Sham)	3.77	12.01	13.74	25.75	4.01	11.75	14.39	26.14	101	106
3 wk Reinn	3.7	11.58	13.87	25.45	4.42	7.39	7.03	14.42	57	120
3 wk Reinn	3.08	10.03	12.65	22.68	3.88	6.32	7.42	13.74	61	126
12 wk Ligate	4.58	12.12	14.5	26.62	5.38	3.92	4.64	8.56	32	117
12 wk Ligate	2.87	11.34	13.1	24.44	3.84	4.03	4.9	8.93	37	134
12 wk Reinn	4.53	12.12	13.82	25.94	5.26	11.26	14.1	25.36	98	116
12 wk Reinn	2.5	4.19	10.05	14.24	2.83	6.71	7	13.71	96	113
1 year Reinn (E1)	3.31	10.91	12.14	23.05	3.61	8.78	10.62	19.4	84	109
1 year Reinn (E2)	3.16	10.89	12.62	23.51	3.15	10.76	10.01	20.77	88	100
1 year Reinn (E3)	4.78	14.8	17.94	32.74	5.36	15.4	17.3	32.7	100	112

heteronymous stretch reflex in reinnervated muscle due to stretch of a reinnervated synergist at the one year time point, well after reinnervation is complete (Foehring et al., 1986a). In this particular protocol one reinnervated muscle is the conditioning stimulus, while the other reinnervated muscle is the test stimulus (Figure 3.4A). Normally, stretch of either G muscle produces additional reflex contraction when stretched together with the other G muscle (Nichols, 1989, 1999). In the case of E1 MG conditioning of LG, the LG in the right (untreated) leg had become inactive at that point in the experiment. As such a comparison could not be made between the right and left legs and this pairing was excluded from the analysis. For all 5 cases, reinnervated conditioning of reinnervated muscle was ineffective in producing a heteronymous stretch reflex (Figure 3.4B-C), with the amplitudes listed in Table 3.2 (Reinn Conditioning of Reinn). LG conditioning of MG produced reflexes that were 3% or less than the control side, the vast majority of which was not associated with EMG activity (Figure 3.4B). MG conditioning of LG also produced very little heteronymous stretch reflex force, with responses less than 18% of the control side (Figure 3.4C; see Table 3.2, Reinn Conditioning of Reinn). All decreases shown were statistically significant ( $p < 0.001$ , Student's t-test). The addition of sural reflex did not increase the amount of heteronymous stretch reflex force (Figure 3.4D) and was a consistent finding for all cases of reinnervated conditioning of reinnervated muscle. We therefore conclude that reinnervated muscle is inefficient in eliciting heteronymous stretch reflexes in regenerated synergist motor pools.

It is entirely possible that the observed ineffectiveness of regenerated Ia afferents to elicit heteronymous stretch reflexes could be restricted to regenerated motoneurons. This is because synaptic stripping occurs on regenerated motoneurons (Blinzinger and Kreutzberg, 1968; Brannstrom and Kellerth, 1998, 1999; Chen, 1978; Linda et al., 2000; Sumner, 1975, 1976). This possibility was tested by examining the ability of reinnervated

**Figure 3.4** *Reinnervated muscle is ineffective in eliciting heteronymous stretch reflexes in regenerated motor pools.* A: Isometric force, length, and EMG traces for MG conditioning of LG during quiescence in the treated leg. Black bars underneath LG force trace indicate conditioned test response. B-C: Reinnervated conditioning of reinnervated muscle. Means and SE during quiescence of heteronymous dynamic reflex force (Heteron Dynamic Reflex) for each cat for MG as conditioning stimulus (B) and LG as conditioning stimulus (C). Asterisks mark statistical significance ( $p < 0.001$ ) between right and left leg (independent Student's t-test). E: experiment number. R: Right Leg; L: Left Leg. Number of trials (n) and mean  $\pm$  SE are shown in Table 3.2 (Reinn conditioned of Reinn). D: Isometric force trace of LG during sural nerve stimulation (X-ext Stim) with MG conditioning indicated by horizontal black bars.



**Table 3.2 *Heteronymous dynamic reflex force amplitudes among the triceps surae.***

Heteronymous dynamic reflex force amplitudes (conditioned test response minus test response) are reported as means  $\pm$  SE and calculated from the number of trials (n) identified, where a single trial consists of one sequential pair of test and conditioned test responses. Means were compared between the untreated (right) leg and the treated (left leg) and the treated mean was expressed as a percentage of the untreated mean (%). All comparisons were significant according to Student's t-test with  $p < 0.001$ . One case had  $p < 0.005$  and is denoted with an asterisk. In E1, movement artifact caused the mean of SOL conditioning of LG to be negative when no EMG was present in the LG in the test or conditioned test responses (see text), hence the SOL was deemed to have no effect on LG and the percentage was considered to be zero (F). Reinn: reinnervated muscle. Spared: injury-spared muscle. E: experiment number.

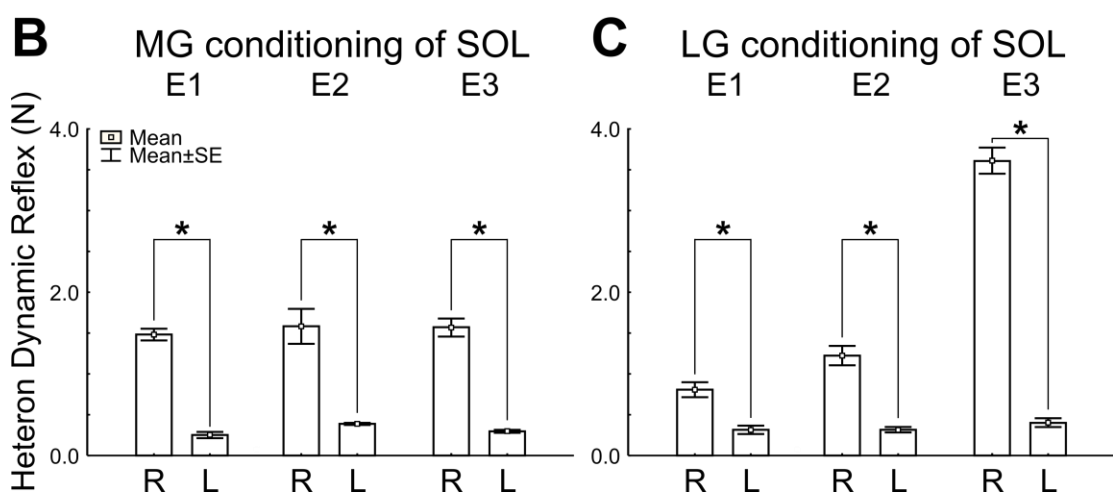
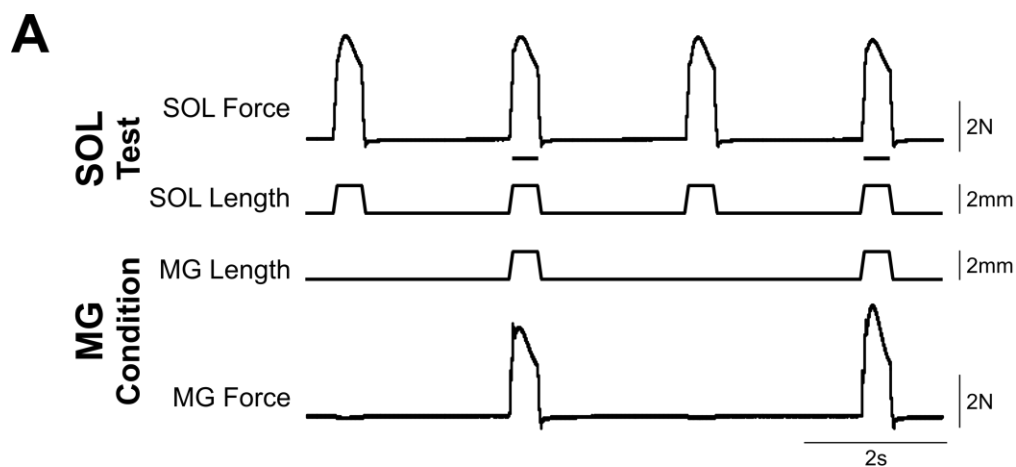


<b>Heteronymous Dynamic Reflex Force (N)</b>							
	<b>Treated Leg (Left)</b>			<b>Untreated Leg (Right)</b>			<b>%R</b>
<b>Reinn conditioning of Reinn</b>	n	Mean	SE	n	Mean	SE	
MG conditioning of LG							
E1	53	4.61	0.34	38	189.90	10.49	2.43
E2	81	15.28	0.85	11	514.95	24.84	2.97
E3	34	9.75	2.77	43	689.32	24.44	1.41
LG conditioning of MG							
E1	n/a	n/a	n/a	n/a	n/a	n/a	n/a
E2	64	65.86	5.93	23	518.14	26.07	12.71
E3	26	101.20	19.69	46	565.90	41.41	17.88
<b>Reinn conditioning of Spared</b>							
MG conditioning of SOL							
E1	16	25.13	3.83	44	148.18	7.22	16.96
E2	99	38.78	1.30	5	158.45	21.45	24.48
E3	98	29.67	1.81	54	157.10	11.03	18.89
LG conditioning of SOL							
E1	11	31.52	5.13	30	80.79	9.17	39.01
E2	23	31.67	3.35	7	122.33	11.83	25.89
E3	17	40.06	5.69	29	360.73	16.03	11.11
<b>Spared conditioning of Reinn</b>							
SOL conditioning of MG							
E1	38	6.97	1.06	16	77.71	12.34	8.97
E2	38	191.24	4.36	30	522.96	22.22	36.57
E3	74	144.01	6.39	37	209.26	28.63	68.82
SOL conditioning of LG							
E1	24	-7.97	0.67	24	42.20	4.27	0.00*
E2	94	24.30	1.00	35	308.02	11.40	7.89
E3	n/a	n/a	n/a	n/a	n/a	n/a	n/a

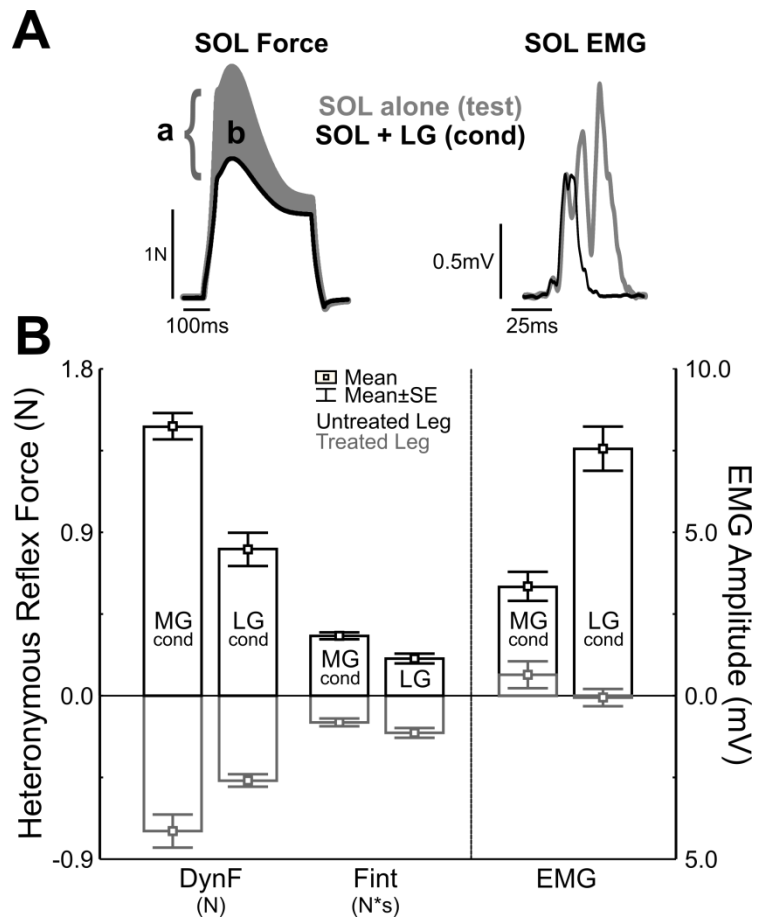
muscle to elicit heteronymous stretch reflexes in an injury-spared synergist. In this particular protocol either the MG or LG is the conditioning stimulus, while the SOL is the test stimulus (Figure 3.5A). The conditioned SOL responses in the quiescent state are shown in Figure 3.5 B-C. Again, it is clear that the response of SOL to either reinnervated MG or LG conditioning is also dramatically decreased, with responses averaging only 23% of the control leg ( $p < 0.001$  for all cases, Student's t-test; see Table 3.2, Reinn Conditioning of Spared). These results confirm and extend those from previous studies (Cope et al., 1994). Therefore the ineffectiveness of regenerated Ia afferents to elicit heteronymous stretch reflexes extends to all synergist motoneurons, both regenerated and injury-spared.

In one cat (E1), stretch of either reinnervated muscle under quiescent conditions produced inhibition of SOL stretch reflexes that was not present on the control side. An example of the inhibition from reinnervated LG conditioning is shown in Figure 3.6A. Inhibition occurred in 83% of quiescent LG conditioning trials and in 65% of quiescent MG conditioning trials. Figure 3.6B demonstrates that this inhibition was reflected in both the heteronymous dynamic force (LG conditioning:  $-46.80 \pm 3.46$ ,  $n=54$ ; MG conditioning:  $-74.52 \pm 9.08$ ,  $n=30$ ) as well as the heteronymous force integral (LG conditioning:  $-10.93 \pm 3.46$ ,  $n=54$ ; MG conditioning:  $-14.67 \pm 2.13$ ,  $n=30$ ). The amount of inhibition was too large to be accounted for by any movement artifact and was often reflected in the EMG (Figure 3.6B, far right). In order to compare the amount of inhibition expressed in each of the force parameters, the inhibition was expressed as a percentage of control excitation (e.g., absolute value of left heteronymous dynamic force mean /right heteronymous dynamic force mean  $\times 100$ ). The amount of inhibition in heteronymous dynamic force and force integral were equally affected

**Figure 3.5** *Reinnervated muscle is ineffective in eliciting heteronymous stretch reflexes in injury-spared motor pools.* A: Isometric force and length traces for MG conditioning of SOL during quiescence in the treated leg. Black bars underneath SOL force trace indicate conditioned test response. It should be noted that a reflex appears in the reinnervated MG force trace due to the simultaneous stretch with the injury-spared SOL. B-C: Reinnervated conditioning of spared muscle. Means and SE during quiescence of heteronymous dynamic reflex force (Heteron Dynamic Reflex) for each cat for MG as conditioning stimulus (B) and LG as conditioning stimulus (C). Asterisks mark statistical significance ( $p < 0.001$ ) between right and left leg (independent Student's t-test). E= experiment number. R: Right Leg; L: Left Leg. Number of trials (n) and mean  $\pm$  SE are shown in Table 3.2 (Reinn conditioning of Spared).



**Figure 3.6 *Reinnervated muscles actively inhibit the injury-spared SOL during quiescence in one cat (E1).*** A: Isometric force traces (left) and rectified and integrated EMG (right) from E1 of the injury-spared SOL while stretched alone (test response, gray) or simultaneously with the reinnervated LG (conditioned test response, black) while at rest. EMG has been filtered for clarity. Heteronymous reflex forces were examined in the heteronymous dynamic force (a) as well as in the heteronymous force integral (shaded area, b). B: Mean and SE comparing SOL heteronymous reflexes (conditioned test minus test) due to conditioning stretch of either the MG (MG cond) or the LG (LG cond) between the untreated leg (right leg, black) and the treated leg (left leg, gray) for dynamic force (DynF), force integral (Fint) and EMG. Points below the zero line indicate inhibition.

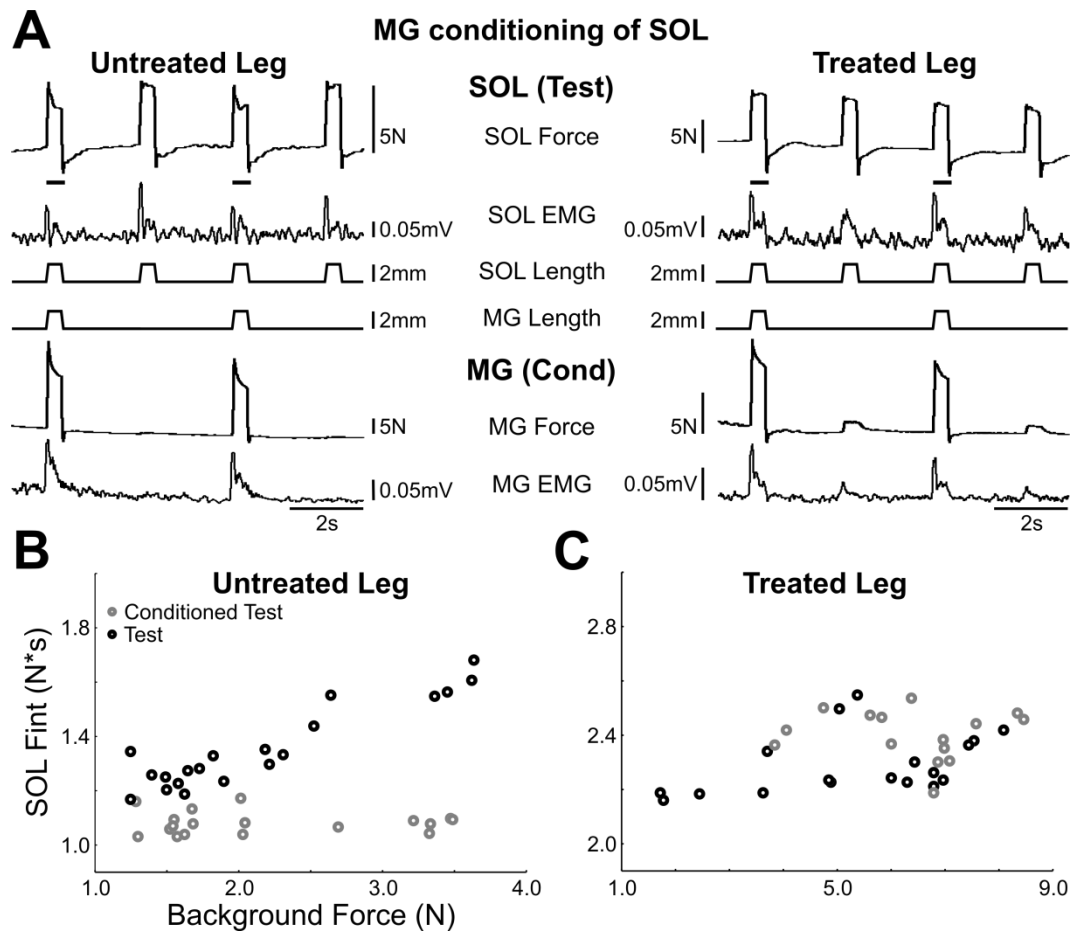


with both MG as conditioning stimulus (50% versus 44%) and LG as conditioning stimulus (58% versus 60%). The inhibition was only present during quiescence and disappeared during the active state at higher background force levels. This observation is in stark contrast to clasp-knife inhibition, where inhibition increases with increased muscle force output (Cleland and Rymer, 1990).

The addition of excitatory drive by eliciting either crossed extension or sural reflexes had no effect on the conditioned SOL response due to stretch of either reinnervated G muscle. Specifically, a lack of force-dependent inhibition was noted in all cats during the active state. An example of MG conditioning of the SOL from a single cat during sural nerve stimulation is shown in Figure 3.7. Normally, stretch of either G produces prominent force-dependent inhibition (Figure 3.7A, left). As it was rare that we saw sustained background forces greater than 7 N in G for either leg, the net inhibition was plotted as a function of the force integral. This is because inhibition of dynamic responses occurs at 9N or more of background force in G (2 N SOL force during sural reflex) while net inhibition is detectable around 5N of G force (1.2 N SOL force during sural reflex; Nichols, 1999). Figure 3.7B demonstrates the occurrence of inhibition in the untreated leg at SOL forces as low as 1.25 N. However, after reinnervation this effect is completely absent (Figure 3.7A, right) and holds true for a wide range of background forces (Figure 3.7C). This loss cannot be explained by lack of force achieved in the G during crossed extension or sural nerve reflexes (cf. Figure 3.3B). These observations suggest that areflexia extends to other forms of proprioceptive input, confirming and expanding results of previous study (Cope et al., 1994).

**Figure 3.7** *Reinnervated muscle fails to produce force-dependent inhibition onto injury-spared muscle.* A: Isometric force, length, and EMG (rectified and integrated) traces from E2 of MG (conditioning stimulus) and SOL (test stimulus) in the untreated (left) and treated (right) legs during sural nerve stimulation at matched background forces (5N). EMG was rectified, integrated, and filtered for clarity. Black bars underneath SOL force trace indicate conditioned test response. Isometric responses in the reinnervated MG due to SOL stretch occurred only during the active state and only on the treated side. B-C: Plot of force integral (Fint) vs. background force for both test responses (black circles) and conditioned test responses (gray circles) of SOL in response to MG stretch in the untreated (B) and treated (C) leg for the same cat.

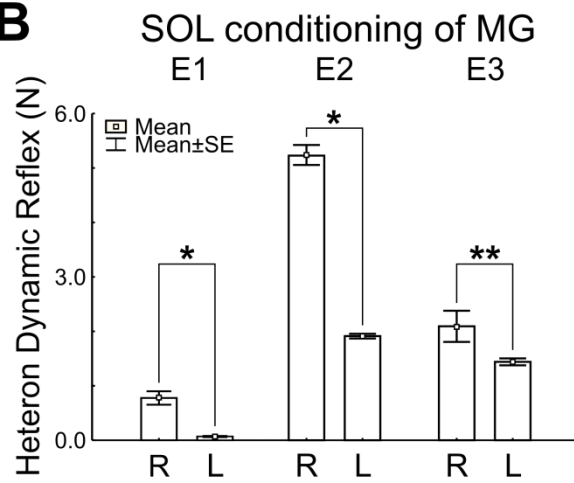
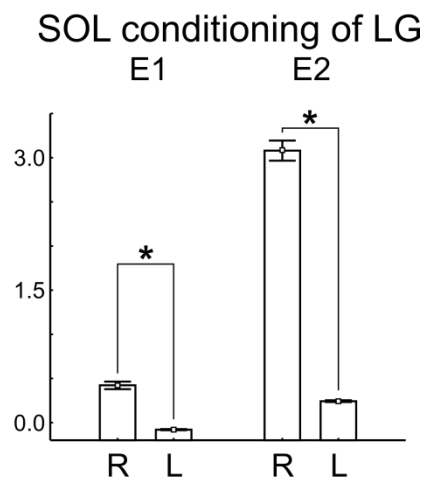
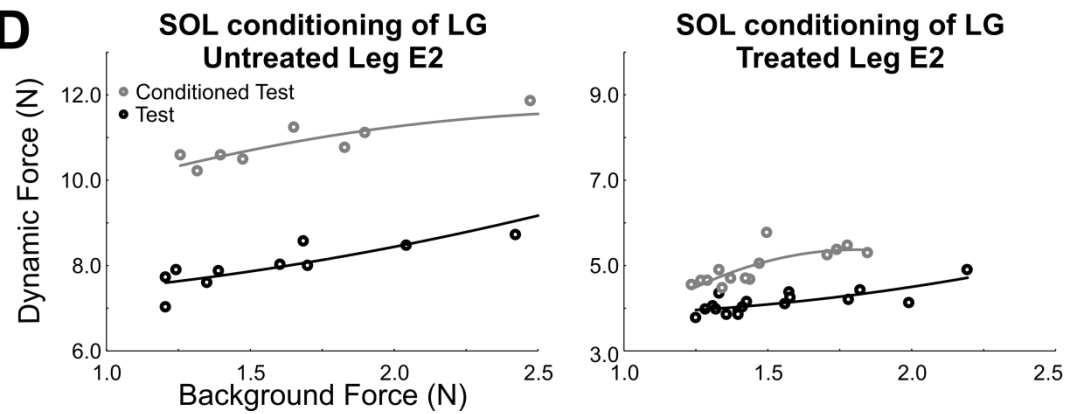




*Injury-spared SOL heteronymous input is decreased after synergist reinnervation*

In order to further examine the interactions among the triceps surae after selective reinnervation, the ability of the injury-spared SOL to initiate heteronymous stretch reflexes in the reinnervated G muscles was tested. On one hand, these heteronymous reflexes may decrease due to synaptic stripping of the regenerated motoneuron. On the other hand, they may increase in order to compensate for lost feedback and force in the reinnervated G muscles. In order to resolve this issue, heteronymous stretch reflexes were collected as detailed in the Methods section and examined in each of the three experiments conducted at the one-year time point. In this particular protocol SOL is the conditioning stimulus, while either the MG or the LG is the test stimulus (Figure 3.8A). Normally, stretch of the SOL produces additional reflex contraction when stretched together with either of the G muscles (Nichols, 1999). Although the amount of heteronymous reflex due to stretch of the injury-spared SOL was variable under quiescent conditions, it is clear that stretch of the injury-spared SOL produced decreased reflex contraction in either of the reinnervated G muscles in the treated leg when compared with the untreated leg (Figure 3.8B-C). In the case of E3 SOL conditioning of LG, the LG in the right (untreated) leg was no longer active at that point in the experiment. As such a comparison could not be made between the right and left legs and this pairing was excluded from the analysis. The mean  $\pm$  SE and the number of trials (n) used to calculate them are listed in Table 3.2 (Spared Conditioning of Reinn). All pairings were significantly decreased in the treated (left) leg when compared to the untreated (right) leg, with 4 out of 5 having a significance of  $p < 0.001$  (Student's t-test). MG conditioned by SOL in E3 had a significance level of  $p < 0.005$ . Interestingly, this experiment had a reinnervated MG that produced a very small amount of reflex, although it was only 1% of the untreated MG (see above). It was determined that the negative mean heteronymous dynamic reflex force shown in E1 for LG conditioned by SOL in the treated leg was not true inhibition

**Figure 3.8 *Injury-spared muscle is less effective in eliciting heteronymous stretch reflexes in regenerated motor pools.*** A: Isometric force, length, and EMG traces from experiment 2 (E2) of MG (test stimulus) and SOL (conditioning stimulus) in the treated leg during quiescence. Black bars underneath MG force trace indicate conditioned test response. B-C: SOL conditioning of reinnervated muscle. Means and SEM during quiescence of heteronymous dynamic reflex force for each cat for MG as test stimulus (B) and LG as test stimulus (C). Single asterisks mark statistical significance of  $p < 0.001$  and double asterisks mark  $p < 0.005$  between right and left leg (independent Student's t-test). E: experiment number. R: Right Leg; L: Left Leg. Number of trials (n) and mean  $\pm$  SE are shown in Table 3.2 (Spared conditioning of Reinn). D: Dynamic force plotted against background for SOL conditioning of LG for E2 during sural reflex stimulation. Test (black circles, LG stretched alone) and conditioned test (gray circles, LG and SOL stretched together) responses are shown for the untreated leg (right leg, left panel) and treated leg (left leg, right panel). Lines represent best fit polynomials and are shown for clarity. Note that the scale is the same for both plots.

**A****B****C****D**

and was actually due to movement artifact for three reasons. First, the test response of the reinnervated LG was indistinguishable from the passive response. Second, this test response was not associated with any EMG production. Third, it was noted that stretch of the SOL while LG was held isometric produced a negative force deflection of about 0.10N, which is approximately equal to the negative mean for the heteronymous dynamic reflex (0.08N). Therefore, stretch of the injury-spared SOL was considered to have no effect on the reinnervated LG in E1. In sum, the injury-spared SOL had a decreased effectiveness in eliciting heteronymous SRs in reinnervated muscle during quiescence.

The addition of crossed extension or sural reflexes did not increase the heteronymous reflex force to control levels in 4 out of 5 cases. Although quantitative analysis during crossed extension proved difficult (see above), a qualitative comparison could often be made between untreated and treated legs. An example of this is shown in Figure 3.8D. When dynamic force is plotted against background force between test and conditioned test responses, a rough estimation of the heteronymous contribution of the injury-spared SOL could be made. It is clear that the difference between test and conditioned test responses is much less in the treated leg (Figure 3.8D, right) when compared to the untreated leg (Figure 3.8D, left), and this observation held for 4 out of the 5 cases analyzed. The exception was for E3 where MG was conditioned by SOL. In this case the difference between test and conditioned test responses was indistinguishable between right and left legs (data not shown). It should be noted that this combination in this experiment produced the largest amount of heteronymous reflex during quiescence when compared with the untreated leg. An interesting observation was that stretch of the injury-spared SOL produced isometric contraction in both reinnervated muscles during the active state (FIG 3.7A, right). This phenomenon was not observed on the contralateral side. Therefore we conclude that even though injury-spared SOL heteronymous

stretch reflexes are present, they are decreased and are not a source of compensation for lost feedback and force from reinnervated synergists.

*Injury-Spared SOL homonymous SRs are reduced after synergist reinnervation*

The observation that the injury-spared SOL had a decreased effectiveness in eliciting heteronymous stretch reflexes in regenerated synergists could be due to post-synaptic factors such as synaptic stripping. However, this decrease could also be explained by a decreased effectiveness of injury-spared afferents in eliciting stretch reflexes. This could be tested directly by examining the ability of injury-spared afferents in eliciting homonymous stretch reflexes, thus avoiding stretch reflex circuitry that had been directly injured and subsequently undergone regeneration. Therefore the SOL homonymous reflex data obtained at the one year time point was combined with a separate study in which SOL homonymous stretch reflexes were examined at different time points according to the process of reinnervation (Foehring et al., 1986b). This study was originally designed to test whether injury-spared homonymous reflexes change in response to loss of feedback and force from the reinnervated G muscles. However, the viability of the SOL nerve is important as it is closely joined to the LG nerve and could have been damaged during survival surgery, thereby affecting our results. To ensure no damage was done, SOL nerves from the treated leg in all cats except one (one 12-wk reinnervated cat) were submitted for histological analysis after termination of the experiment (see Methods). All nerves were found to have intact axons and myelin sheaths indistinguishable from normal (Figure 3.9C).

We observed unequivocal stretch reflexes in all injury-spared SOL at all time points during the quiescent state, regardless of treatment. A typical example of the injury-spared SOL

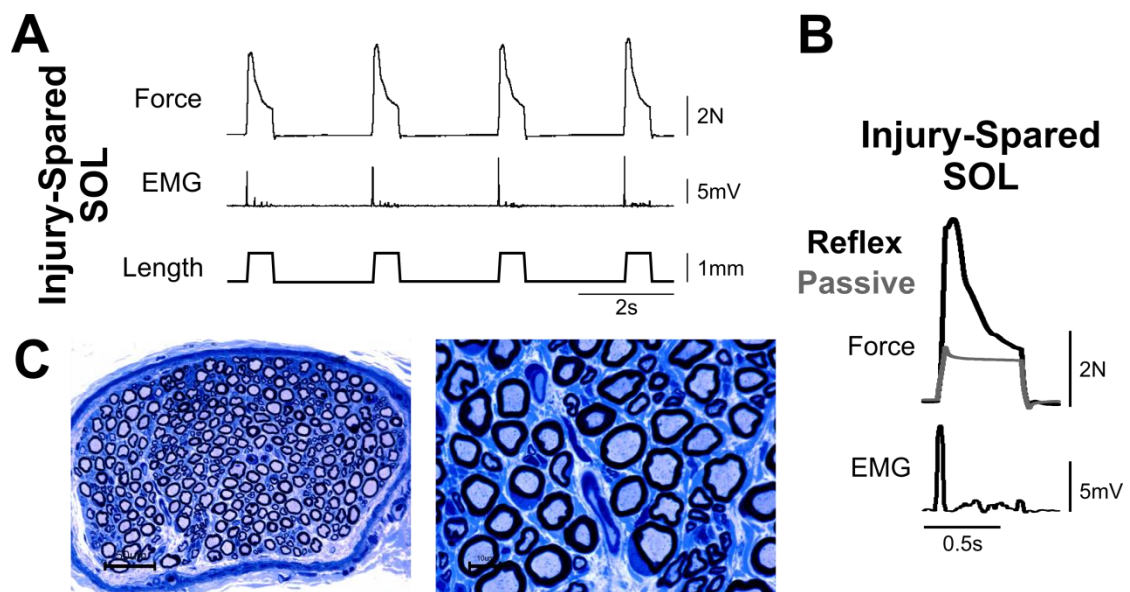
response to stretch is shown in Figure 3.9(A-B). SOL force responses to ramp hold release stretches were recorded under quiescent conditions at resting length throughout multiple trials. A prominent force response to stretch (black trace, reflex plus passive response) was expressed in the dynamic force when compared to the passive response taken with the SOL nerve cut (gray trace; Figure 3.9B). This observation is in direct contrast to previous work which reported that SOL homonymous stretch reflexes were lost after reinnervation of the MG and LG muscles, even during conditions of additional excitation (Maas et al., 2007).

SOL homonymous stretch reflexes at all time points were then compared to control animals. In order to make accurate comparisons across the entire group, stretch reflex amplitudes were normalized for muscle weight. Homonymous stretch reflexes in the sham-treated animal were between the ranges of the two controls (Figure 3.5A, filled square) so all three animals were grouped together as a control group. Comparison between the control and treated groups show a clear trend in relation to the process of reinnervation. This data is presented in Figure 3.10A, with the corresponding mean  $\pm$  SE and the number of trials (n) used to calculate them listed in Table 3.3. The data collected suggests that SOL homonymous stretch reflexes are decreased if reinnervation has occurred and either remained the same or were elevated if it did not. Given the variability among groups, data could not be pooled to create average means. Individual data points were therefore compared to either the minimum or maximum control value.

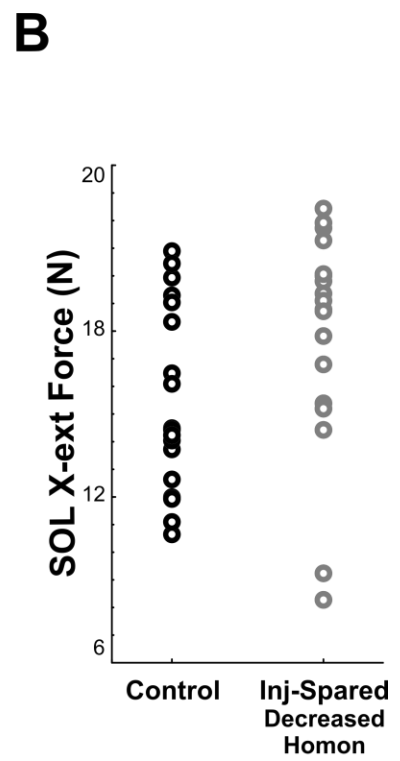
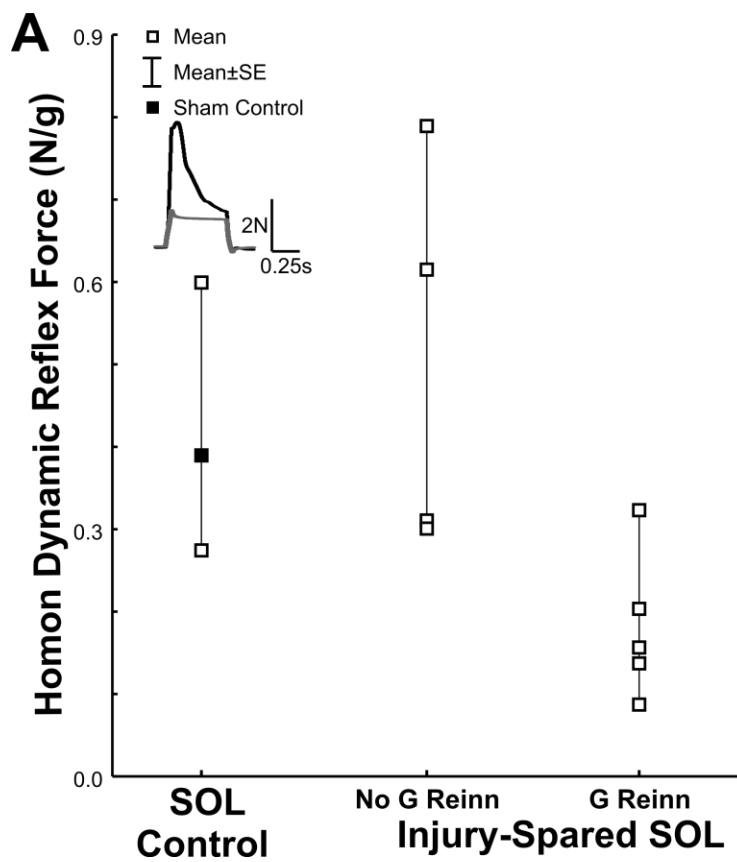
First, the group consisting of non-reinnervated animals (3 week Reinn and 12 week Ligate) was compared with the control group. At three weeks after nerve section and reunion, the MG and LG are effectively denervated as reinnervation has not yet begun (Foehring et al., 1986b). In order to confirm this during the terminal experiments, the ipsilateral tibial nerve

**Figure 3.9 *Injury-spared synergist produces homonymous stretch reflexes.*** A: Isometric force, EMG and length trace for injury-spared SOL homonymous test responses to ramp hold release stretch obtained 3 weeks after section and surgical union of MG and LG nerves. B: Isometric force and EMG (rectified, integrated, and filtered for clarity) traces of an injury-spared SOL homonymous stretch reflexes (black) overlaid onto the passive (gray) response in the treated leg during the quiescent state. Passive response did not produce EMG when stretched. C: SOL axons undamaged by surgical section of nerves to close synergists (MG and LG) performed 3 weeks previous to terminal experiment. Toluidine blue stained cross sections of the injury spared SOL nerve from the same cat (20x, left, 50um scale bar; 60x, right, 10um scale bar) showing intact axons and myelin sheaths indistinguishable from normal. Histology provided by Dr. Charlotte Sumner.





**Figure 3.10 *Reinnervation has an adverse affect on spared SOL reflex magnitude.*** A: Status of spared SOL homonymous reflex at various time points with respect to synergist reinnervation (see Methods). Homonymous stretch reflex amplitude was calculated by passive subtraction from recorded dynamic force. Data shown for homonymous stretch reflexes during quiescence from the left leg for all experiments. Filled square in control column indicates sham control. Reinn: reinnervated. Inset: isometric force traces EMG (rectified, integrated, and filtered for clarity) of an injury-spared SOL homonymous stretch reflexes (black) overlaid onto the passive (gray) response in the treated leg from a single cat during the quiescent state. B: Amount of force produced by SOL in response to crossed extension or sural stimulation (SOL X-ext Force) for control and cases where the injury-spared SOL had decreased homonymous reflexes (Inj-Spared Decreased Homon). The 4 largest force responses were plotted for each experiment (3 control, 4 injury-spared).



**Table 3.3** *Injury-spared SOL homonymous dynamic reflex force at various time points after synergist treatment.* SOL homonymous dynamic reflex force amplitudes are reported as mean  $\pm$  SE and calculated from the number of trials (n) identified, where a single trial consists of a single response to ramp hold release stretch.

Treatment	Left SOL Homon Dyn Reflex	
	n	Mean $\pm$ SE
Control	230	0.2743 $\pm$ 0.0060
Control	146	0.5994 $\pm$ 0.0125
Control (Sham)	246	0.3895 $\pm$ 0.0061
3 wk Reinn	367	0.3007 $\pm$ 0.0035
3 wk Reinn	82	0.7891 $\pm$ 0.0112
12 wk Ligate	177	0.3106 $\pm$ 0.0027
12 wk Ligate	227	0.6149 $\pm$ 0.0071
12 wk Reinn	502	0.2033 $\pm$ 0.0024
12 wk Reinn	269	0.1372 $\pm$ 0.0036
1 year Reinn (E1)	68	0.1564 $\pm$ 0.0102
1 year Reinn (E2)	61	0.3232 $\pm$ 0.0120
1 year Reinn (E3)	61	0.0872 $\pm$ 0.0049

proximal to triceps surae nerve branches was electrically stimulated. This stimulation did not elicit contraction in either the MG or LG muscle on the treated side even though the SOL was contracting strongly. Furthermore, G muscle weights were only 61% or less than the untreated side (cf. Table 3.1). At this time point, the SOL homonymous stretch reflex was found to be either within the control range or increased by approximately 30%. In order to determine whether the process of reinnervation itself would be responsible for any changes in the SOL homonymous stretch reflex, reinnervation was prevented by ligation of the MG and LG and homonymous stretch reflexes were studied 12 weeks later. Again, lack of reinnervation was confirmed during the terminal experiments by stimulation of the ipsilateral tibial nerve and G muscle weights were only 37% or less than the untreated side. 12 weeks after synergist ligation, the same pattern was observed as for the 3 week reinnervated group: both experiments fell within the control range. Although we cannot determine if the injury-spared SOL homonymous stretch reflex is increased if reinnervation has not occurred due to half the data points falling within control levels and half being increased, the following observation is clear: the injury-spared SOL homonymous stretch reflex is not decreased.

In the group where reinnervation was allowed to occur, the picture changes quite dramatically. By 12 weeks, the MG and LG have started to regain force production as reinnervation is well underway. At this time point, reinnervated G muscle weights were within 4% of the untreated side. One experiment was 75% of the lowest control value, while the other was only 50%. This difference is not attributable to differences in SOL weight as the SOL on the treated leg in both animals was at least 113% of the untreated leg (cf. Table 3.1). Lastly, we allowed full reinnervation of synergists to occur by allowing one year to pass from the time of nerve section and reunion. Two out of three cats showed decreased homonymous SRs at 57% and 32% of the lowest control, while the third was within the

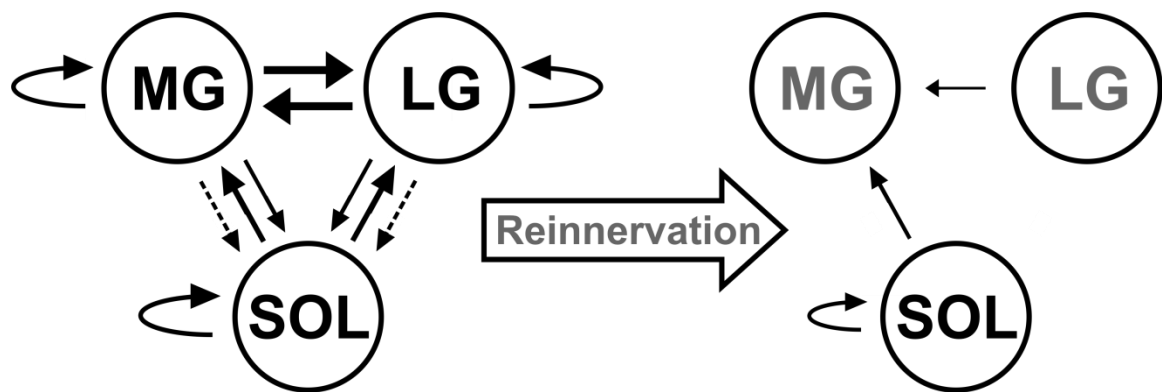
control range. Therefore if reinnervation is allowed to occur, 4 out of 5 cats demonstrated decreased injury-spared SOL homonymous stretch reflexes. This decrease was not due to the ability of SOL motoneurons to respond to other sources of excitatory input, as they produce similar amounts of force in response to crossed extension or sural reflex when compared to controls (Figure 3.10B). We conclude that when reinnervation of synergists is allowed to occur, homonymous stretch reflexes in the injury-spared SOL are decreased. Furthermore, this decrease clearly indicates that injury-spared homonymous stretch reflexes do not compensate for lost feedback and force in reinnervated muscles.

## **Discussion**

Our primary objective in this study was to examine the functionality of heteronymous projections of regenerated Ia afferents among a group of synergists, one of which was injury spared. We identified a number of factors that influence stretch reflex amplitude. Despite controlling for these factors, we found that after peripheral nerve regeneration, coordination among the triceps surae is practically obliterated, with muscles acting in relative isolation of proprioceptive feedback (Figure 3.11, right). This is in sharp contrast to the normally tight coordination mediated through the projections of proprioceptive afferents (Eccles et al., 1957; Nichols 1989; Nichols 1999), and these projections are summarized in Figure 3.11 (left). Heteronymous stretch reflexes from reinnervated muscle were found to be dramatically decreased in both regenerated and injury-spared motor pools. This ineffectiveness fits the view that peripherally regenerated primary afferents retract their central axonal collaterals from spinal cord regions occupied by motor pools (Alvarez et al., 2011). We also found that both homonymous and heteronymous stretch reflexes from the injury spared SOL are decreased in relation to synergist reinnervation. The decreased effectiveness of length feedback from injury-spared muscle, both homonymous and

**Figure 3.11** *Synergist muscles become disordinated after reinnervation.* Schematic of muscle-length feedback distributed among the triceps surae muscles before (left) and after (right) selective nerve injury and reinnervation. Curved arrows: homonymous reflex; solid arrows: excitatory heteronymous reflex; dashed arrows: heteronymous force-dependent inhibition. Arrows are scaled to reflect differences in magnitude.





heteronymous, may suggest that afferent retraction extends to afferents of injury-spared synergists. Furthermore, decreases in stretch reflexes from injury-spared muscle suggests that increases in motor pool excitation that mediate compensation through injury-spared muscle must necessarily come from other sources.

*Lost heteronymous stretch reflexes mediated by regenerated afferents*

In the present study, the status of heteronymous stretch reflexes from reinnervated muscle onto a synergist motor pools, both regenerated and injury-spared, confirms and extends our earlier observation (Cope et al., 1994) that these stretch reflexes are dramatically reduced or lost. Previous effort has emphasized investigating mechanisms through which homonymous stretch areflexia occurs and these findings shed light on and support current hypotheses.

While there are certainly changes in motoneuron excitability after injury in both cats (Kuno et al., 1974a; Foehring et al., 1986; Gustafsson and Pinter, 1984; Mendell et al., 1995) and rats (Bichler et al., 2007; Gardiner and Seburn, 1997; Nakanishi et al., 2005; Yamuy et al., 1992), the ineffectiveness of heteronymous stretch reflexes mediated by regenerated Ia afferents onto injury-spared motor pools suggests that areflexia may be predominantly accounted for by presynaptic, rather than postsynaptic, mechanisms. This makes sense as intrinsic motoneuron properties seem to be restored after reinnervation in both the cat (Kuno et al., 1974; Foehring et al., 1986; Mendell et al., 1995) and the rat (Bichler et al., 2004; Bullinger et al., 2011a; Haftel et al., 2005). Furthermore, present results confirm that motoneurons are capable of responding to other sources of excitatory input (Cope et al., 1994). With considerations of the post-synaptic side accounted for, we next turn our attention to presynaptic mechanisms for the loss of heteronymous stretch reflexes elicited by regenerated Ia afferents.

From a presynaptic standpoint, approximately 50% of regenerated primary afferents respond to stretch (Collins et al., 1986), and reinnervation of inappropriate targets by muscle afferents has also been known to occur (Brushart 2011). Furthermore, it has been shown physiologically that some regenerated primary afferents make functional connections with motoneurons but are not sensitive to stretch (Bullinger et al., 2011a). Therefore inappropriate reinnervation of peripheral targets certainly plays a role in the observed stretch areflexia. However, the majority of afferents that are sensitive to stretch fail to make functional connections with regenerated homonymous motoneurons (Bullinger et al., 2011a). This is supported anatomically by the loss of VGLUT1 in lamina IX and the retraction of regenerated primary afferents from LIX in the spinal cord (Alvarez et al., 2011), which also indicates a loss of synaptic connections with motoneurons in synergist motor pools. The evidence presented in this report gives functional evidence that regenerated Ia afferents retract from all motoneurons, both homonymous and heteronymous, regardless of injury. Therefore even if regenerated Ia afferents make appropriate connections in the periphery, it is not enough to restore the stretch reflex. Still, a combination of synaptic disassembly and failure of peripheral connections would explain the loss of heteronymous stretch reflexes from reinnervated muscle.

The presence of stretch-evoked inhibition from reinnervated muscles seen in one cat is an intriguing phenomenon. It is clear that the inhibition requires stretch of the reinnervated G. The most likely source of inhibition would be from extrinsic neural circuits, possibly the same ones postulated to suppress homonymous stretch reflexes in reinnervated muscle (Haftel et al., 2005). One postsynaptic source of inhibition may be from Ib afferents (Jami 1992). However, the normal force-dependent inhibition seen at higher forces from G onto SOL was completely absent in from reinnervated muscle which suggests these regenerated

afferents may be affected by the same processes as described above. What is more likely is a presynaptic mechanism that affects the regenerated afferents directly. One form of presynaptic inhibition, called primary afferent depolarization (PAD), has been shown to be sustained (Enriquez-Denton et al., 2004) and enhanced (Enriquez et al., 1996) several weeks after nerve crush in the cat. In order for this mechanism to work, however, PAD must be initiated by stretch of reinnervated muscle as it usually occurs tonically. It remains to be shown whether PAD is present after reinnervation and is capable of being activated by stretch.

#### *Decreased stretch reflexes mediated by injury-spared afferents*

Although we found that injury-spared SOL stretch reflexes are decreased in relation to synergist reflexes that are lost and never regained, two issues must be addressed. First, evaluation of our hypotheses required the presence of injury-spared SOL stretch reflexes. According to a previous report, SOL homonymous stretch reflexes were found to be lost after reinnervation of the G muscles, even during conditions of additional excitation (Maas et al., 2007). Although reduced, we still observed unequivocal homonymous and in most cases heteronymous stretch reflexes initiated by injury-spared muscle. There for although this study agrees qualitatively with the results of Maas et al., there is still a quantitative difference. The most likely explanation for this discrepancy is that the animals used in the Mass et al. studies were heavily instrumented with EMG. This could have impaired the muscle's ability to produce a reflex, possibly through some sort of immunological response. Second, while heteronymous stretch reflexes from injury-spared muscle remained relatively intact, they were nonetheless significantly reduced. Previous electrophysiological studies at the Ia-motoneuron synapse indicate normal (Eccles et al., 1962) or increased (Mendell et al., 1995) EPSP amplitude in regenerated motoneurons when elicited by electrical stimulation of

injury-spared nerves. Therefore we might expect these reflexes to be completely maintained. The maintenance of EPSPs versus the lack of eSSPs in regenerated homonymous circuits was attributed to afferents that retained central connectivity with motoneurons but failed to make appropriate peripheral connections (Bullinger et al., 2011a). This explanation, however, would only be specific to regenerated afferents as those that are injury-spared do not lose connectivity with their peripheral mechanoreceptors. Since the EPSP amplitudes reported above reflect the afferent population as a whole, it could be that those injury-spared afferents that retain their connections with motoneurons, e.g. do not retract, have EPSP amplitudes that remain normal or are increased. However, this remaining connectivity is still not enough to restore the stretch reflex. Further investigation of single Ia-motoneuron connectivity of injury-spared Ia afferent connections is needed to resolve this issue.

The decreased stretch reflexes initiated by injury-spared Ia afferents may be caused by a similar disconnect between Ia afferents and motoneurons observed to occur with regenerated Ia afferents. This afferent retraction, however, occurs as a result of the initial injury as VGLUT1 immunoreactivity is dramatically decreased one week after tibial nerve ligation or reinnervation, and fails to return to normal levels long after reinnervation has occurred (Alvarez et al., 2011). In our time course study of injury-spared homonymous stretch reflexes, decreased stretch reflexes only occurred if reinnervation was allowed to occur. The presence of stretch reflex amplitude similar to or above control values in the absence of reinnervation would suggest that the decreased stretch reflexes from injury-spared muscle are due to the process of synergist reinnervation and not the injury itself. Therefore if these afferents do retract or have decreased synaptic contacts with motoneurons in lamina IX, it is due to a separate signal related to synergist reinnervation. These observations still do not rule out the presence of an extrinsic inhibitory circuit that would suppress the actions of the

stretch reflex circuit as we originally postulated (Haftel et al., 2005). In fact, the decreased stretch reflexes from injury-spared muscle would seem to support this idea. It may even be the same circuit as the one proposed to mediate the case of inhibition from reinnervated muscle described above, as both would necessarily be stretch-activated.

The decrease in stretch reflexes initiated by injury-spared muscle therefore does not explain the recovery of kinematics in level/upslope walking (Maas et al 2007). However, this may be expected due to the lower contributions of length feedback under these conditions (Nichols and Houk, 1976; Huyghues-Despointes et al., 2003a,b). It also cannot explain the partial recovery in downslope walking (Abelew et al, 2000; Maas et al 2007), a condition where the triceps surae is subjected to greater changes in length during contraction (greater eccentric contraction) and is thus more dependent on proprioceptive feedback. Therefore adaptations in locomotion due to increased force output in spared muscles occurring at 3 weeks or later must be attributable to other sources. We now turn our attention to plausible candidates for the source of increased motoneuronal drive to injury-spared motor pools.

#### *Possible candidate sources of excitatory drive to injury-spared motoneurons*

One peripheral source of increased motoneuronal drive is increased feedback from cutaneous inputs. While cutaneous inputs from the plantar surface of the paw may be more critical in challenging locomotor tasks than in locomotor pattern generation, there is evidence they may contribute to excitatory levels in motoneurons. For example, it has been shown that cutaneous feedback from the plantar surface of the paw in premamillary cats may contribute to background firing in muscle, thereby increasing or decreasing muscular response to perturbations (Honeycutt and Nichols, 2011). They have also been shown to modulate motor unit firing during fictive locomotion (Degtyarenko et al., 1996). Furthermore, tibial nerve

reflexes have been shown to be enhanced after muscle denervation (Frigon and Rossignol, 2007). Therefore it is possible that this enhanced feedback could increase background firing in muscle, thereby increasing excitatory drive to motor pools. Whether this excitation is adequate in strength or selectivity of activation to account for spared synergist compensations remains to be determined.

Yet another peripheral source of compensation might be enhanced positive force-feedback pathways during locomotion. At rest, force feedback mediated by Ib afferents supplying Golgi tendon organs has been well-documented in mediating non-reciprocal group I inhibition (Eccles et al., 1957b; Granit 1950; Granit and Sursoet, 1949; Hunt 1952; Laporte and Lloyd, 1952; Sherrington 1909). However, this Ib-mediated inhibitory force feedback has been observed to switch to excitatory during locomotion (Angel et al., 1996; Conway et al., 1987; Gossard et al., 1994; McCrea et al., 1995). Indeed, these pathways have been shown to contribute to muscle activity during locomotion in the cat (Pearson and Collins, 1993; Donelan and Pearson, 2004; Gregor et al., 2006; Donelan et al., 2009), although its distribution appears to be much more limited than previously shown (Ross and Nichols, 2009). Furthermore, Ib afferents project widely throughout the hindlimb (for review see Jami, 1992), and may even account for the increases in activity seen in multiple muscles throughout the hindlimb (Frigon and Rossignol, 2007). Therefore modest enhancements of positive force feedback may contribute to injury-spared motor pools.

A central source of compensation could come from an increase in central locomotor drive. This is consistent with parallel increases in both early and late EMG components seen after denervation (Bouyer et al., 2001, Gritsenko et al., 2001, Frigon and Rossignol, 2007), as the early component is thought to be generated centrally through the locomotor pattern generator

(Engberg & Lundberg, 1969; Gorassini et al., 1994). This may also explain the preservation of kinematics after ankle extensor reinnervation as originally postulated by Abelew et al. (2000). It is likely that signals from descending inputs may increase excitatory drive as cats that recovered locomotion after flexor denervation experienced substantial deficits after subsequent spinalization (Carrier et al., 1997). It is important to recognize, however, that trained treadmill-walking cats that were first spinalized then denervated were able to compensate for the loss of denervated muscles (Carrier et al., 1997; Frigon and Rossignol, 2008), suggesting that the spinal cord is capable of mediating limited compensations on its own.

### *Summary*

In sum, the results presented here suggest that stretch reflex dysfunction after peripheral nerve regeneration is not limited to regenerated homonymous pathways. Stretch areflexia of reinnervated muscles extends to heteronymous motor pools, and stretch reflex dysfunction even extends to an injury-spared synergist. These findings may account for the inability for locomotor deficits in interjoint coordination to fully recover during downslope walking after reinnervation of the triceps surae (Abelew et al., 2000; Maas et al., 2007). The extent of areflexia would lead us to predict that additional abnormalities would be found during other conditions relying on lengthening contractions, such as running or regaining balance after unexpected perturbations. Furthermore, the results presented here are in line with our current view that regenerated afferent retract their central axon collaterals from spinal cord regions occupied by motor pools. Two questions still remain. First, does stretch reflex dysfunction extend to other Ia-mediated pathways, such as those coordinating antagonist actions? Second, what are the mechanisms behind afferent retraction seen after peripheral nerve injury and regeneration?



## **CHAPTER 4: Reorganization of stretch-activated antagonist reflexes after reinnervation**

### **Introduction**

With the finding in Chapter 3 that muscles reinnervated by severed nerves are ineffective in generating stretch reflexes in not only the homonymous muscle but also in injury-spared synergists, we next turn our attention to other projections of Ia afferents. An additional pathway through which these afferents also transmit length feedback is through the reciprocal inhibitory circuit. Normally, Ia afferents also send off collaterals that initiate inhibition of antagonist motor pools. This is accomplished through a disynaptic pathway (Araki et al., 1960; Eccles et al., 1956; Eccles and Lundberg, 1958; Jankowska and Roberts, 1972) with an interposed inhibitory premotor interneuron located in lamina VII of the spinal cord (Hultborn et al, 1976b; Jankowska and Lindström, 1972). This reciprocal organization allows for coordinated movement of mutual antagonists about a joint so that corrections to postural disturbances may occur unimpeded. This pathway may also be involved in coordinating flexor and extensor activity during locomotion (Geertsen et al., 2011). Due to this organization, it is easy to see how increases in reciprocal inhibition would support out-of-phase activity of antagonist muscles during locomotion, while decreases in reciprocal inhibition would promote co-contraction. The functional status of this pathway after reinnervation, however, remains unknown.

Recent study by Alvarez et al. (2011) provides anatomical clues as to the status of reciprocal inhibition after peripheral nerve regeneration. Through analysis of the number of VGLUT1 contacts, they demonstrated that regenerated primary afferents retain some synapses in spinal cord laminae V and VII. Although smaller in size, these contacts were redistributed, with the number of contacts in lamina VII remaining similar to controls while the number of contacts in lamina V increased by over two-fold. The apparent maintenance of contacts in lamina VII would therefore suggest that synapses between Ia afferents and premotor interneurons are largely retained. This is in sharp contrast to the loss of VGLUT1 immunoreactivity in lamina IX. These observations provide compelling evidence that some proprioceptive feedback pathways may be lost, while others may be retained. However, the functional state of those synapses that appear to be maintained remains unknown. Taken together, these findings led us to examine whether regenerated stretch-sensitive afferents were also impaired in producing reflexes between muscle antagonists, e.g. reciprocal inhibition.

Despite the retention of synapses in lamina VII, available literature suggests that reciprocal pathways may nonetheless be disrupted after reinnervation. Recent study by Sabatier et al. (2011) suggests that after reinnervation, there is an increase in the incidence of co-contraction of antagonists during locomotion. After cut and surgical repair of the sciatic nerve, EMG activity indicated that SOL was active throughout the step cycle, not just during the stance phase. This was also observed for the TA, which showed EMG activity throughout both the swing phase (when it is normally active) and the stance phase. This change in EMG activity was also observed with a more selective surgical treatment. Instead of treating the entire sciatic nerve, the branches of the common peroneal (including the branch to the TA) and the tibial nerve (including the branches supplying SOL) were individually cut and surgically rejoined. This was performed in order to decrease the amount

of inappropriate reinnervation of the muscles under study. The authors concluded that since coactivation of TA occurred under both experimental conditions, this coactivation was centrally generated as opposed to being the result of axonal misdirection. The experiments just described involved study of reciprocal activation between two muscles that are both reinnervated. Only one study to date has examined reciprocal activation patterns after a more selective reinnervation (Wasserschaff, 1990). In this series of experiments, only the common peroneal nerve was cut and surgically rejoined in mice, and EMG activity was recorded 14 weeks after transection. As with the study described above, there was an increase in coactivation between the reinnervated TA and the uninjured SOL. The presence of co-contraction in each of these studies would therefore suggest that reciprocal inhibition is decreased after reinnervation. Although these EMG studies provide clues that these pathways may be disrupted, no studies to date have directly tested the functionality of reciprocal inhibitory pathways after reinnervation.

In order to resolve these issues, the objective of this study was to directly test the effectiveness of length feedback from a reinnervated antagonist by directly measuring muscle force output in response to stretch. The common peroneal nerve supplying the tibialis anterior (TA) was cut and surgically rejoined. Heteronymous stretch reflexes were then measured from the TA onto the injury-spared gastrocnemius (G; medial and lateral gastrocnemius together) muscle in vivo long after reinnervation had been established. We found that after reinnervation, there was shift from net inhibition to net excitation of the injury-spared muscle due to reinnervated antagonist stretch. This suggests a profound reorganization of spinal circuitry after peripheral nerve regeneration. Preliminary results have been presented in abstract form (Cope and Horstman, 2011).

## **Methods**

### *Animals*

Data were obtained in 14 adult female Wistar rats aged 11-18 months (Charles Rivers Laboratories, Wilmington, MA), ranging in weight from 340-480 g. Rats were separated into two groups, either treated or control. Rats in the treated group underwent a chronic nerve treatment applied in a survival surgery. All rats were studied during single terminal experiments, after which they were euthanized by isoflurane overdose and exsanguination.

### *Survival Surgery*

In order to study changes in antagonist reflexes after peripheral nerve regeneration, animals in the treated group underwent nerve treatment performed during a survival surgery. This was performed in a dedicated surgical suite using sterile techniques. Anesthesia was induced and maintained throughout the surgical procedure (1.0-2.5% in 100% oxygen) via inhaled isoflurane through a nose cone. Once corneal and withdrawal reflexes were absent, a skin incision was made over the popliteal fossa of the left hindlimb to gain access to the nerves under study. The left common peroneal nerve (including the branch supplying the TA) was isolated using blunt dissection. After isolation, the nerve was sectioned and surgically rejoined end to end through the epineurium with one to two sutures using 10-0 ethilon. The wound was irrigated with 0.9 % saline and individual fat, fascial and skin layers were sutured closed. A subcutaneous injection of buprenorphine (0.1 mg/kg) was given before anesthesia was discontinued. Animals were returned to their cages upon discontinuation of anesthesia and were monitored throughout the recovery period. Additional buprenorphine was given every 12 hours for the next 48 hours to alleviate pain and animals were monitored for signs of stress or infection. All animals received regularly scheduled veterinary care until the time

of terminal surgery. The animals were then studied in terminal experiments 9-14 months after undergoing nerve treatment.

*Terminal Experiment: Anesthesia and Surgical preparation*

Anesthesia was induced by inhaled isoflurane in an induction chamber (4-5% in 100% oxygen). Animals were intubated for terminal experiments in order to maintain a patent airway, after which anesthesia was maintained through a tracheal cannula (1-3% isoflurane in 100% oxygen). The carotid arteries were then ligated bilaterally for decerebration later in the experiment. In order to ensure that deep anesthesia was maintained throughout the terminal experiment, heart rate, blood O<sub>2</sub> saturation, expired CO<sub>2</sub>, temperature ( $37 \pm 1^\circ \text{C}$ ), and respiratory rate were monitored. Vital signs were maintained through the adjustment of isoflurane concentration, adjustment of radiant and water-pad heat sources, and administration of Ringer-dextrose solution (1 mL/hr). Withdrawal reflexes were also monitored to ensure they remained suppressed.

The TA and G muscles and nerves were each isolated bilaterally. Resting lengths of each muscle corresponding to 90° flexion of the knee and ankle were marked with suture.

Tendons were cut while retaining a piece of the calcaneus at the end of each tendon. The posterior tibial nerve and the caudal cutaneous sural nerve (sural nerve) were isolated bilaterally and ipsilaterally, respectively, and cut to allow for stimulation. A plastic nerve cuff fitted with bipolar electrodes were secured around contralateral posterior tibial nerve and the ipsilateral sural nerve was mounted on bipolar electrodes for stimulation later in the experiment (see below). The animal was then moved to a stereotaxic frame where the head, spine and hips were secured. Hindlimbs were secured with bone pins just above the knee and the ankle was secured with a clamp (approximately 90° at both the knee and ankle) attached

to the mechanical ground by magnetic bases. One pair of fine-wire electrodes was inserted into the TA and each head of the G (MG and LG separately) for EMG recording. Since reflex contraction of muscle is suppressed by isoflurane, the animal was rendered insensate by performing a decerebration. This was accomplished by removing all brain tissue rostral to a mid-collicular transection. Gaseous anesthesia could then be discontinued in order to study whole muscle response to stretch. All vital sign monitoring continued until the end of the experiment when the cat was euthanized (see above).

### *Muscle Reflex Assessment*

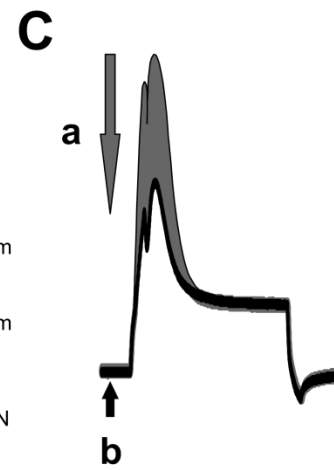
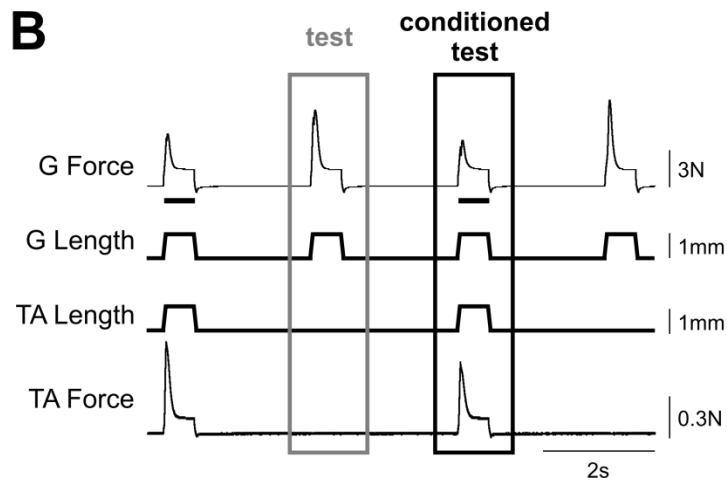
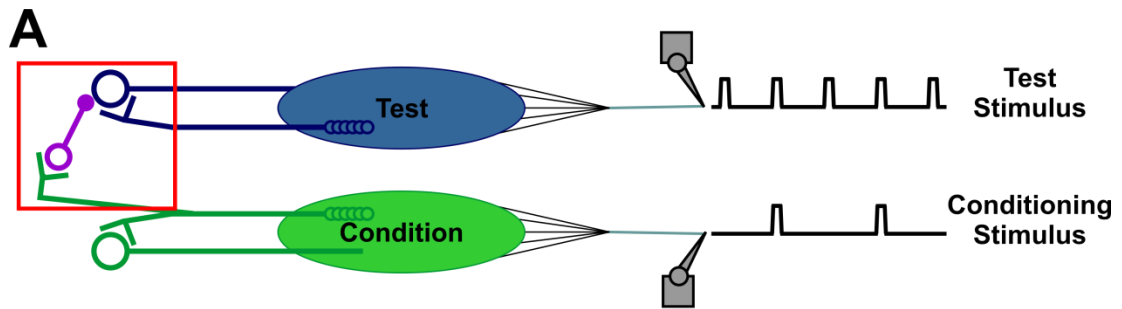
Both homonymous and heteronymous muscle reflexes were assessed as described previously (Huyghues-Despointes et al., 2003a; Nichols, 1999). Briefly, muscle tendons were tied to force transducers in parallel with length-servo motors controlled by customized software. Muscle force, muscle length, and EMG were recorded, digitized (20 kHz) and stored on a computer for later analysis using CED Spike 2 software. Reflexes were evoked by ramp-hold-release stretches using ramp duration = 50ms, amplitude = 1mm, and hold period = 500ms. Muscles were stretched at 2s intervals in order to decrease history dependence yet still maximize data collection at elevated force levels. Stretches were applied when the muscle was inactive (quiescent state). Muscles were also activated by electrical stimulation (100Hz) of either the ipsilateral caudal cutaneous nerve or the contralateral posterior tibial nerve through the crossed extensor reflex. Because force responses to either of these inputs were not sustained long enough to make accurate ramp comparisons, stretches were performed over a range of background forces achieved by adjusting the motor lever which increased the passive force experienced by the muscle. Since force responses to stretch contain both a intrinsic component due to the non-reflexive mechanical properties of muscle in addition to the reflexive component (Nichols and Houk, 1976), the passive intrinsic

component (passive response) was estimated at the end of each experiment by stretching the muscles after the tibial nerve was cut. In order to assess the force-generating capacity of the TA in relation to muscle weight and reinnervation, the maximum tetanic tension (tetanic force) was obtained after passive response collection by recording the maximum force response to electrical stimulation (50Hz) of the cut tibial nerve.

Homonymous and heteronymous stretch-evoked reflexes were assessed as detailed in Chapter 2 and will be briefly summarized here. Homonymous stretch reflexes were assessed by stretching a single muscle alone where the background force was subtracted from the force measured at the peak of the ramp stretch (dynamic force). The force directly attributable to reflex was calculated by subtraction of the passive response and is referred to as the homonymous dynamic reflex. Antagonist reflex interactions were studied by conditioning the stretch reflex of the G (test stimulus) muscle by stretching the TA(conditioning stimulus). The stretch protocol and the synapse under study is shown in Figure 4.1A with corresponding control data traces shown in Figure 4.1B. One muscle was stretched on each trial and is termed the test stimulus ("G Test" traces in Figure 4.1B). Force responses to stretch of this muscle alone (test, black box) represent the test response and is comprised of both the homonymous stretch reflex and the intrinsic response. The other muscle was stretched on alternate trials and is termed the conditioning stimulus ("TA Condition" traces in Figure 4.1B). This muscle will be the source of heteronymous input. Stretching the two together results in a conditioned response of the test muscle (conditioned test, gray box), consisting of both homonymous and heteronymous reflex components in addition to the intrinsic response. In order to estimate the heteronymous reflex contribution, the test response was subtracted from the conditioned test response, leaving only the heteronymous contribution from the conditioning muscle.

**Figure 4.1** *Heteronymous stretch-evoked reflex assessment between antagonists.* A: Muscle stretch paradigm and underlying neural circuitry. One muscle was stretched on each trial and is termed the test stimulus (muscle labeled "Test") while the other muscle was stretched on alternate trials and is termed the conditioning stimulus (muscle labeled "Condition"). Open forks: excitatory connection from Ia afferent whose axon innervates muscle spindles. Closed circle: inhibitory connection from premotor interneuron. Large open circles: motoneuron cell body whose axon projects back to muscle. The synapse under study is shown in the red box. B: Raw traces from a control experiment of gastrocnemius (G, test stimulus) and tibialis anterior (TA, conditioning stimulus) during quiescence. The black box is the test response (G stretched alone; G stretch test), while the gray box is the conditioned response (G stretched together with TA; TA stretch conditioned test). The black bar under G force responses indicates conditioning by TA. C: Heteronymous contributions were calculated by subtracting the test response from the conditioned test response. The resulting force difference (shaded gray area) is the heteronymous dynamic reflex due to antagonist stretch (a). The force occurring immediately before stretch is referred to as the background force (b).





These calculations were made for the dynamic force and the resulting force difference is referred to as the heteronymous dynamic reflex (Figure 4.1C, a).

For heteronymous reflex amplitude, a single trial consisted of one sequential pair of test and conditioned test responses. Since the goal of this study was to examine changes in reciprocal inhibition, trials in which the test response did not produce a reflex were excluded. This is because in the case of inhibition, the conditioned test response is smaller than the test response (indicated by the down arrow in Figure 4.1C), resulting in a negative heteronymous dynamic reflex force. If there is no homonymous reflex present, inhibition clearly cannot be detected. In contrast, positive heteronymous dynamic force amplitudes were considered facilitory. It was noted that heteronymous dynamic force amplitudes that fell within 0.1 N of zero were almost always accompanied by no change in the EMG or lacked appreciable differences in the force profile. This was likely caused by variability in passive responses from stretch to stretch, as noted previously in the rat MG muscle (Haftel et al., 2005). This range of uncertainty was also comparable to their estimated error due to this variability ( $\pm 0.1$  N in our study versus approximately  $\pm 0.09$  N in the Haftel study). Therefore heteronymous dynamic forces that fell within 0.1 N of zero were considered to have no difference between the test and conditioned test responses. Given these three sets of responses, heteronymous reflex actions due to antagonist stretch were parsed into the following categories: inhibitory, facilitory, or no change.

In order to selectively activate Ia afferents, heteronymous reflexes were also assessed by subjecting the muscle to vibration in addition to ramp hold release stretch. This is because Ia afferents are exquisitely sensitive to high frequency vibration (De-Doncker et al., 2003; Matthews, 1972). Using the same test-conditioned test paradigm as described above,

muscles were vibrated 80  $\mu$ m at 100 Hz for 5 seconds. There is no intrinsic component to the force response to vibration, so there was no need for passive subtraction. The area under the force profile was calculated (force integral) and used for the assessment of vibration reflexes.

### *Statistics*

Treatment effects were assessed by comparing across treated and control groups. Statistical comparisons were made using nested analysis of variance (nested ANOVA) and Tukey's honestly significant difference (HSD) post hoc test in order to account for differences within groups (SYSTAT, Systat Software, Point Richmond, CA). The post hoc test Fisher's LSD was used to test for significance between mean percentages. Pearson correlations were used to analyze relationships of force profiles. Skewness was calculated for each treatment group in order to interpret differences in data set distribution. The non-parametric Kolmogorov-Smirnov test was used to test for significance in this distribution. Independent Student's t-test was used to assess significance of differences between test and conditioned test responses. Data are reported as mean  $\pm$  SEM unless otherwise noted. The level of significance was set at  $p < 0.05$  for all statistical tests.

## **Results**

### *Factors that influence stretch reflex amplitude*

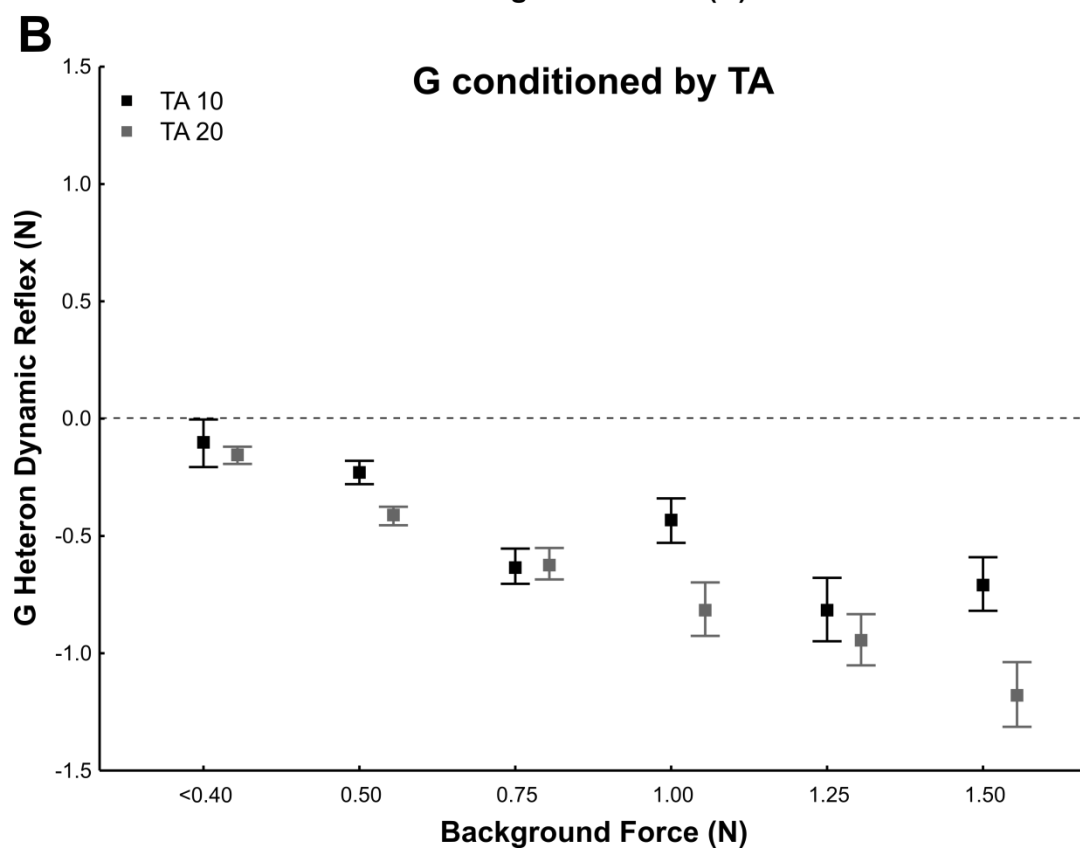
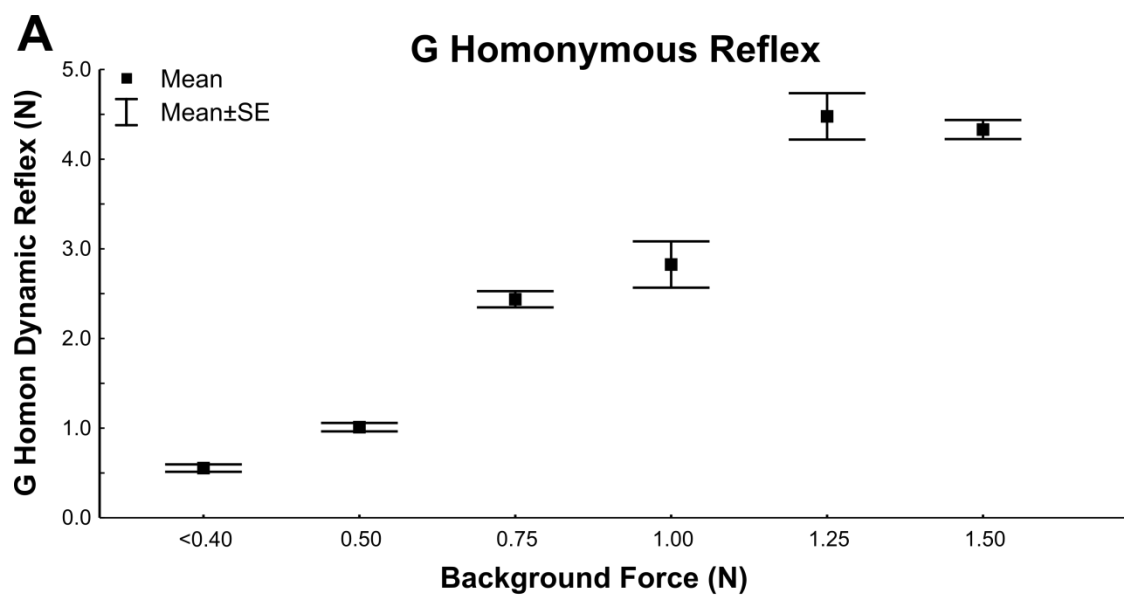
There are a number of factors that can affect reflex force in control animals. Animal weight may have an influence on load bearing and may or may not contribute to reflex force.

Furthermore, stretch reflex amplitude decreases with age as motor unit firing rate is impaired (Erim et al., 1999; Soderberg et al., 1991). These factors were controlled for at the outset by only using animals aged 11-18 months old. The amount of background force also influences the amount of reflex amplitude as larger motor units are progressively recruited (Matthews,

1968). Therefore comparisons of stretch reflex amplitude were made at matched background forces. The amount of background force was modulated in these experiments by adjusting the passive length in order to achieve multiple levels of background force in 0.25 N intervals. The force at resting length of G ranged from 0.1-0.3 N. Therefore responses below 0.4 N were considered as a single background group and the next force tested was 0.5 N. Typical G force responses to ramp hold release stretch in a control rat under quiescent conditions over a range of background forces are shown in Figure 4.2A. In these plots, the mean and SE are shown for ramps held at each of the separate background levels. It is clear that the homonymous reflex force increases with increasing background force.

The amount of background force also influences the amount of heteronymous dynamic reflex force due to stretch of the TA in control animals (Figure 4.3B), with the amount of inhibition increasing with the amount of stretch of G as measured by the amount of G background force (see above). Therefore only experiments which data was collected at a range of background forces under quiescent conditions were included in heteronymous stretch reflex assessment. The amount of passive stretch on the TA may also influence the heteronymous reflex force. Normally, the force at resting length on the TA is 0.05 N. However, this force produced variable conditioned test responses in G (data not shown). Therefore the muscle was stretched out to 2 times resting force and, in some cases, 4 times resting force. It is clear that there is little difference in heteronymous force responses whether the TA was held at 0.1 N or 0.2 N. As such, heteronymous analysis was carried out under conditions where TA resting force was 0.1 N. This was done in order to include the maximum number of experiments in the heteronymous reflex analysis. Due to the increases in both homonymous and heteronymous reflex force with background force, data was collected over a wide range of

**Figure 4.2** *Factors affecting stretch-evoked reflex amplitude.* Amount of reflex force is correlated with background force (A-B). A: Plot of G homonymous dynamic reflex force amplitude (G Homon Dynamic Reflex) versus background force for a control rat during quiescence. Muscle was stretched out in 0.25N force increments to achieve greater levels of background force. The amount of homonymous reflex at each background force level is expressed as the mean and SE. B: Plot of heteronymous dynamic reflex force amplitude (Heteron Dynamic Reflex) versus background force for the same rat under the same conditions with the TA held at 0.1N (black) and 0.2N (gray).



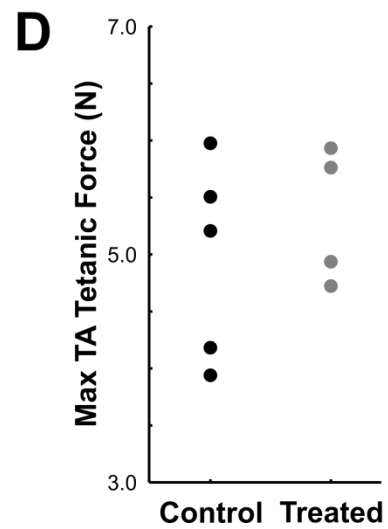
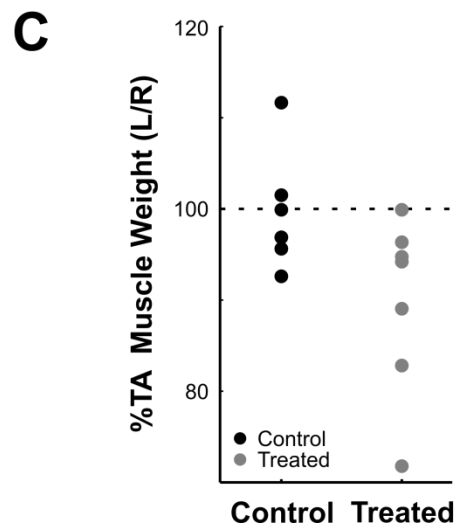
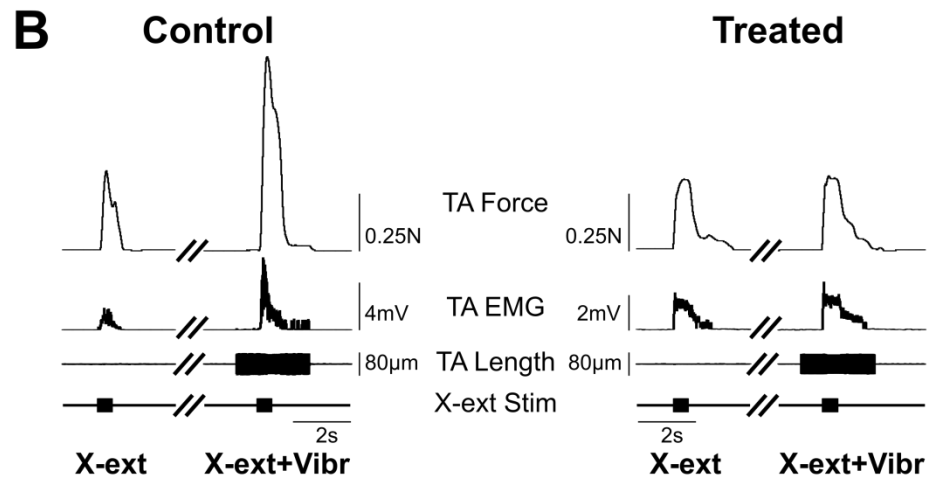
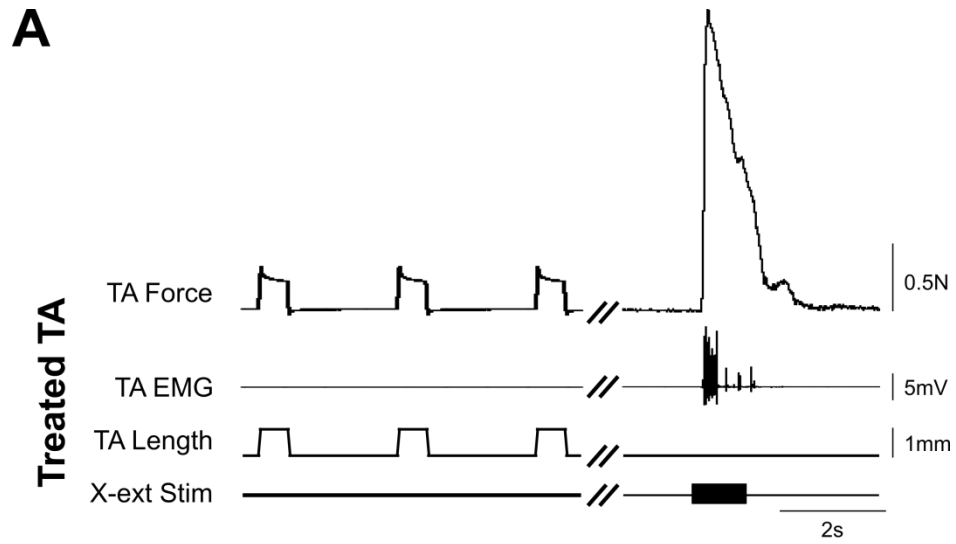
background forces and compared. With these factors that influence stretch reflex amplitude controlled, we can now turn to study of stretch reflexes in animals in which the TA has been reinnervated.

*Reinnervated TA fails to generate force in response to muscle stretch*

One observation that is critical to the success of determining the functional status of reciprocal projections of regenerated Ia afferents is that reinnervated muscles produced little if any reflex force in response to ramp hold release stretch during quiescence (Figure 4.3A). This was determined by both the absence of EMG as well as by comparison to the passive intrinsic response at matched background forces (Table 4.1). This comparison is valid during quiescence as there was no background activity and thus no additional active intrinsic response to be accounted for. This loss of homonymous stretch reflexes is in accordance with previous studies (Cope and Clark, 1994; Cope et al., 1994; Hughues-Despointe et al., 2003; Haftel et al 2005; Maas et al., 2007). TA homonymous reflex forces were collected at 0.1 N in all experiments, so all experiments were included in this portion of the analysis. The homonymous reflex of the test response was incorporated into this data set in order to increase the number of force responses analyzed. The loss of a homonymous stretch reflex held true for all animals in the treated group except one (Experiment 12, see Table 4.1). Data from this experiment was therefore excluded from the main analysis of heteronymous stretch reflexes and was examined separately, the results of which are described at the end of the present report. The lack of a homonymous stretch reflex in reinnervated muscle in 6 out of 7 cases occurs despite the observation that the reinnervated TA surprisingly responded to uninjured sources of excitatory input through crossed extension or sural nerve stimulation (Figure 4.3A, right). Since these force responses typically did not last long enough for

**Figure 4.3** *Status of reinnervated TA.* A: Reinnervated TA fails to produce homonymous SRs despite normal force output. Isometric force, EMG and length trace for reinnervated TA homonymous test responses to ramp hold release stretch. X-ext Stim: crossed extension reflex stimulus. B-C: TA has undergone significant reinnervation. B: Force, length and EMG for a control (left) and treated (rat) during crossed extension alone (X-ext) and during crossed extension superimposed with vibration (X-ext + Vibr). Force traces have been filtered to remove vibration artifact and EMG has been rectified and integrated for clarity. C: TA muscle weight expressed as a percentage of the TA on the contralateral (untreated) side (left TA/right TA) for individual control (black) and treated (gray) animals. D: Maximum tetanic force produced by the TA for individual control (black) and treated (gray) animals. Tetanic forces could not be obtained from earlier experiments due to expiration of the animal.





**Table 4.1** *Homonymous stretch reflex amplitudes of TA and G.* Homonymous dynamic reflex force amplitudes during quiescence for both TA and G in control and treated groups. Reflex amplitudes are reported as mean  $\pm$  SE and calculated from the number of trials (n) identified, where a single trial consists of one sequential pair of test and conditioned test responses. The reinnervated TA expressed no reflex response in all cases except one (marked with asterisk), and this case was examined in a separate analysis. There was no statistical difference in G homonymous stretch reflex amplitudes between control and treated groups ( $p=0.182$ , nested ANOVA with Tukey's HSD).

<b>Homonymous Dynamic Reflex Force (N)</b>					
<b>Group: Control</b>			<b>Treated</b>		
<b>TA</b>			<b>TA</b>		
Exp	n	Mean $\pm$ SE	n	Mean $\pm$ SE	Exp
1	31	0.2142 $\pm$ 0.0108	11	-0.0000 $\pm$ 0.0124	8
2	24	0.1564 $\pm$ 0.0141	48	0.0187 $\pm$ 0.0009	9
3	59	0.4453 $\pm$ 0.0141	50	0.0012 $\pm$ 0.0030	10
4	24	0.2845 $\pm$ 0.0090	37	0.0170 $\pm$ 0.0050	11
5	45	0.3078 $\pm$ 0.0093	35	0.0165 $\pm$ 0.0026	13
6	51	0.1280 $\pm$ 0.0047	35	0.0154 $\pm$ 0.0022	14
7	34	0.1733 $\pm$ 0.0037	64	0.3949 $\pm$ 0.0051	12*
<b>G</b>			<b>G</b>		
Exp	n	Mean $\pm$ SE	n	Mean $\pm$ SE	Exp
3	39	3.8236 $\pm$ 0.1687	43	2.0443 $\pm$ 0.1989	9
4	34	0.9167 $\pm$ 0.0514	32	5.0294 $\pm$ 0.1355	11
5	27	0.7023 $\pm$ 0.0583	32	2.2881 $\pm$ 0.1647	13
6	49	3.0459 $\pm$ 0.1245	54	11.1163 $\pm$ 0.3187	14
7	64	0.8477 $\pm$ 0.0395	50	8.7199 $\pm$ 0.1511	12*

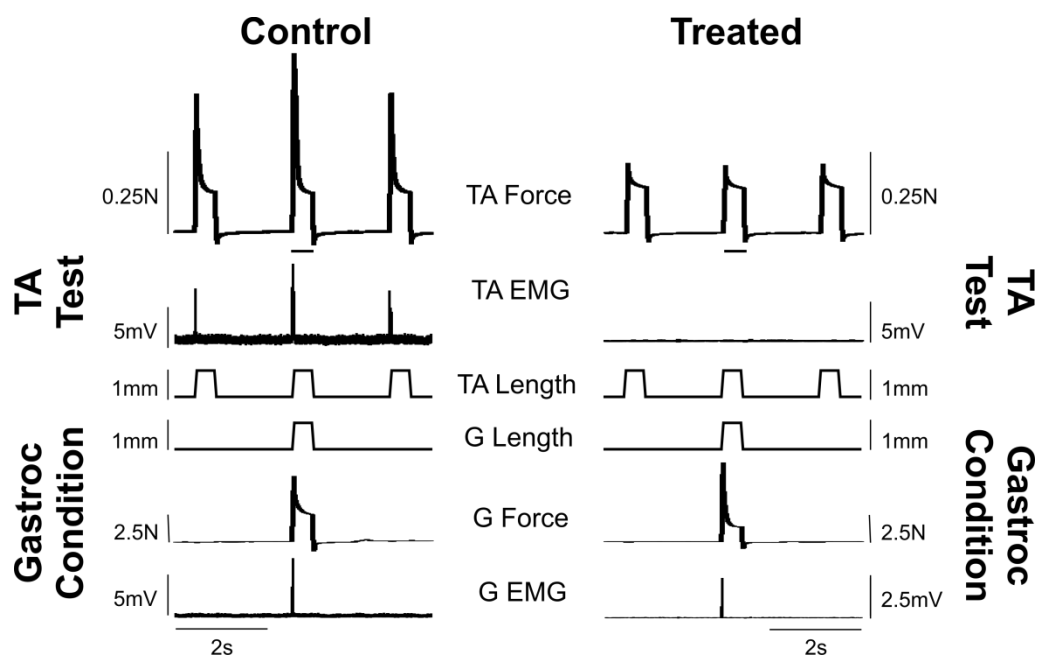
ramp hold release stretches to be superimposed upon them, crossed-extension stimulation was superimposed with vibration, and the resulting force response was compared to the amount of force generated by crossed extension stimulation alone. Normally, the force achieved by the addition of vibration is much greater than crossed extension alone (Figure 4.3B, left). It is clear that the reinnervated TA (Treated, right) produced little if any additional force or EMG when crossed extension stimulation and vibration were combined. This held true for all treated animals that failed to produce reflex in response to ramp hold release stretch. Despite these failures, there was good indication of successful reinnervation of muscle fibers by motoneurons as muscle weights were on the whole within 18% of the untreated side (Figure 4.3C, Table 4.2). Tetanic forces were only obtained in some experiments due to premature expiration of the animal. In experiments where these measurements were obtained, tetanic forces between control and treated TA muscles were also similar (Figure 4.3D) which further indicates substantial reinnervation of muscle fibers by motoneurons.

Reinnervated TA also failed to respond to stretch of the injury-spared G (Figure 4.4). In controls, stretch of G always produced facilitation of TA (Figure 4.4, left). This is in contrast to findings in the cat, where stretch of MG or SOL during quiescence produces inhibition in the TA (Nichols, 1989). The failure of reinnervated TA to respond to stretch of G held true for all treated animals which failed to produce homonymous reflexes in the reinnervated TA. This finding was not due to the lack of G homonymous reflex force as this was present in all treated animals (Table 4.1). It should be noted that two control and two treated animals had to be excluded from analysis of homonymous stretch reflexes in G due to lack of data at sufficient background forces. Furthermore, the amplitude of this reflex was similar between

**Table 4.2 *Muscle weights of G and TA.*** Wet muscle weights for TA and G muscles were obtained in both the right and left legs immediately after each experiment was completed. Muscle weights were expressed as a percentage of the right leg then compared between untreated and treated groups (cf. Figure 4.3C). Asterisk denotes case where the reinnervated TA produced a homonymous reflex.

Treatment	TA Muscle Weight (g)			G Muscle Weight (g)		
	TA Right	TA Left	%TA	G Right	G Left	%G
Control						
1	0.68	0.69	101.47	0.64	0.46	71.88
2	0.68	0.65	95.59	0.7	0.66	94.29
3	0.64	0.62	96.88	0.64	0.57	89.06
4	0.69	0.77	111.59	0.64	0.53	82.81
5	0.68	0.63	92.65	0.55	0.53	96.36
6	0.74	0.74	100.00	0.63	0.63	100.00
7	0.65	0.63	96.92	0.57	0.54	94.74
Treated						
8	0.64	0.46	71.88	1.75	1.63	93.14
9	0.7	0.66	94.29	1.95	1.85	94.87
10	0.64	0.57	89.06	1.73	1.59	91.91
11	0.64	0.53	82.81	1.82	1.66	91.21
13	0.55	0.53	96.36	1.75	1.51	86.29
14	0.63	0.63	100.00	1.87	1.59	85.03
12*	0.57	0.54	94.74	1.59	1.48	93.08

**Figure 4.4** *Reinnervated muscle fails to respond to spared antagonist stretch.* Isometric force, length, and EMG (rectified and integrated) traces of TA (test stimulus) and G (conditioning stimulus) from both a control (left) and a treated (right) animal at matched background forces. The black bars under TA force responses indicate conditioning by G.



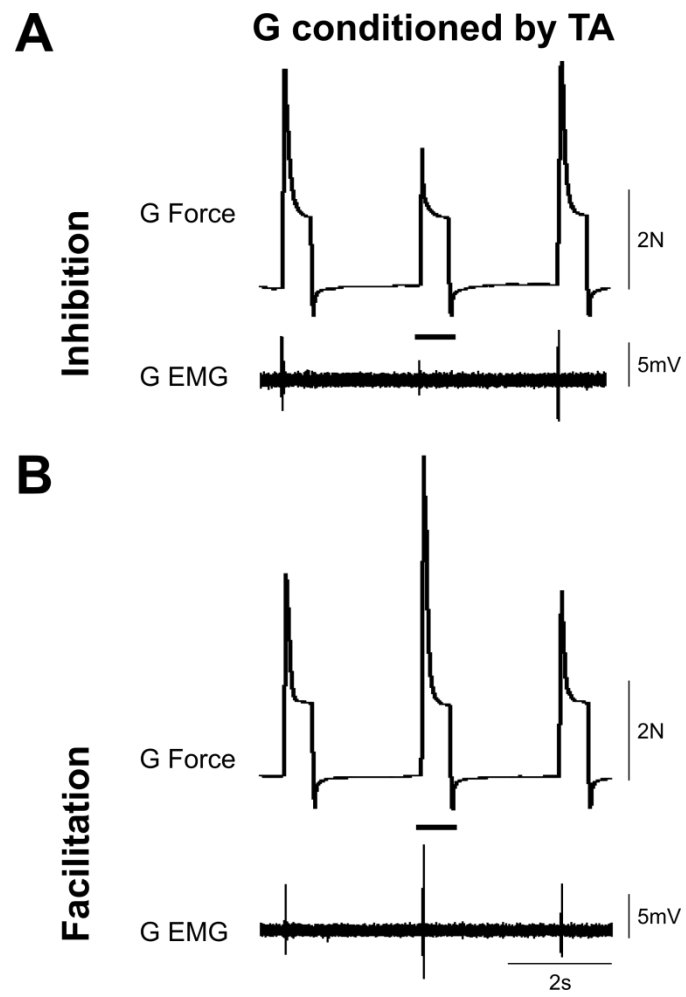


control and treated groups ( $p=0.182$ , nested ANOVA with Tukey's HSD). These findings, taken with those stated above, lead us to the conclusion that reinnervated TA fails to respond to muscle stretch, both homonymous and heteronymous in origin.

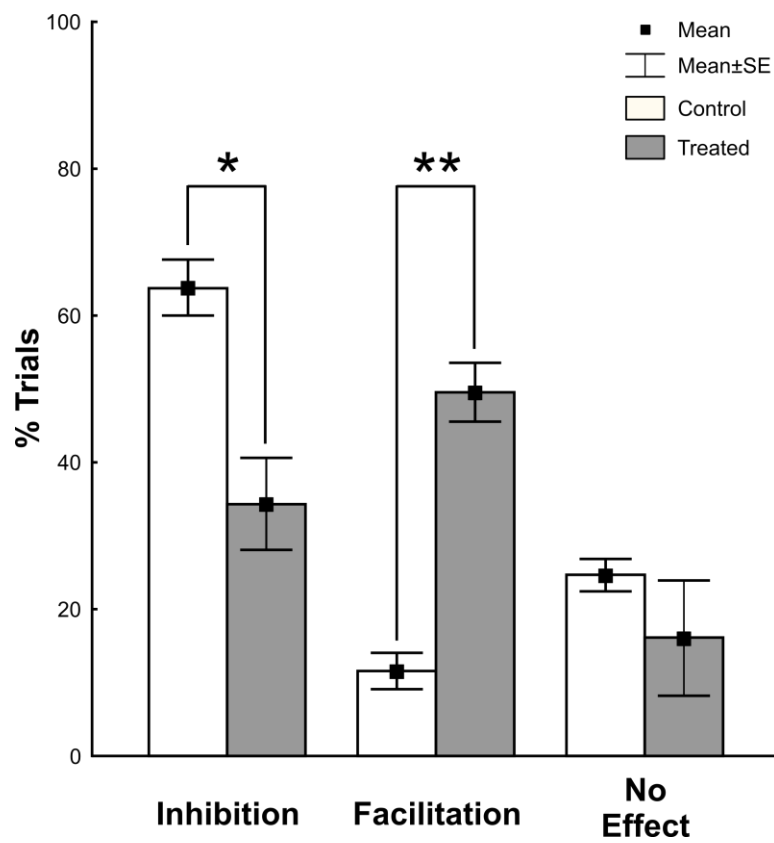
*Reinnervated TA produces stretch-evoked reflexes in an injury-spared antagonist*

With confirmation of the failure of reinnervated muscle to produce homonymous stretch reflexes, we can now turn to an examination of the functionality of circuits mediated by regenerated afferent projections to antagonist motor pools. Two control animals and two treated animals had to be excluded from this analysis due to either insufficient background forces, the presence of tonic activity during higher background forces, or lack of data taken with TA held at 0.1 N. In contrast to our findings in Chapter 3 that reinnervated muscle was ineffective in producing heteronymous stretch reflexes in synergists, stretch of the TA produced substantial heteronymous reflexes in the injury-spared G (Figure 4.5). We were caught by surprise with the finding that conditioning by TA produced a substantial mix of both inhibitory (4.5A) and facilitory (4.5B) responses. These actions were present in the same data collection sequence under the same conditions and were therefore not attributable to fluctuations in excitability of the preparation. In order to get a sense of the frequency of occurrence of these reflex responses, the percentage of trials in which inhibition, facilitation, or no effect occurred (see Methods) was calculated for each of the control ( $n=5$ ) and treated ( $n=4$ ) animals that were included in the analysis. The mean of each group was then compared. These calculations were made over the entire range of background forces that was collected. We found a dramatic redistribution in the frequency of occurrence of inhibition and excitation due to antagonist stretch after reinnervation (Figure 4.6). Significance was calculated using nested ANOVA with Fisher's LSD in order to compare the mean percentage of trials occurring in each reflex category between control and treated groups. In the case of

**Figure 4.5** *Reinnervated TA produces both inhibition and facilitation of G.* Isometric force and EMG of G (test stimulus) from a single treated animal at matched background forces. The black bar under the middle force response indicates it has been conditioned by the reinnervated TA. A: Inhibition of G by conditioning of reinnervated TA. B: Facilitation of G by conditioning of reinnervated TA.



**Figure 4.6 *Redistribution of inhibition and facilitation after reinnervation.*** The percentage of trials in which G experienced inhibition, facilitation, or no change in response to TA conditioning was calculated for individual experiments over a matched range of background forces. One sequential test-conditioned test pair constitutes a single trial. Negative amplitudes of heteronymous dynamic reflex force were inhibitory, while positive values were facilitatory. Trials whose heteronymous dynamic reflex force fell within 0.01 N of zero were equivocal and included in the No Change group (see Methods). The mean percentage was then calculated for both the control group (white bars, n=5) and the treated group (gray bars, n=4) and compared. Single asterisk denotes  $p < 0.005$ , double asterisk denotes  $p < 0.001$  (ANOVA with Fisher's LSD).



inhibition, the percentage of trials decreased by almost half, from 64% to 34%, after reinnervation of TA ( $p < 0.005$ ). In contrast, the percentage of trials expressing facilitation due to TA stretch increased over three-fold, from 12% to 50% ( $p < 0.001$ ). The difference in the frequency of trials in which no appreciable reflex change occurred was not significantly different ( $p = 0.0928$ ). This shift in frequency towards facilitation after reinnervation may therefore reflect a redistribution of Ia inputs to excitatory interneurons.

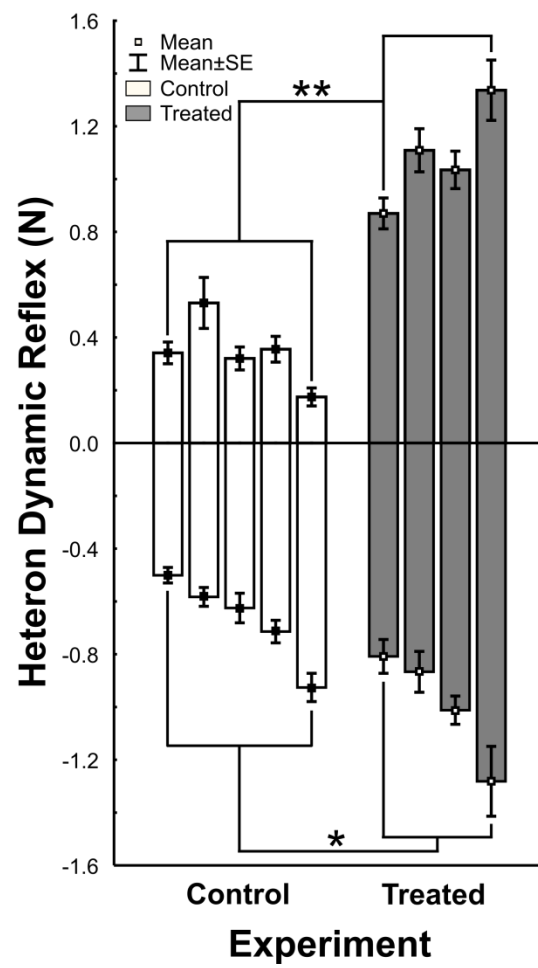
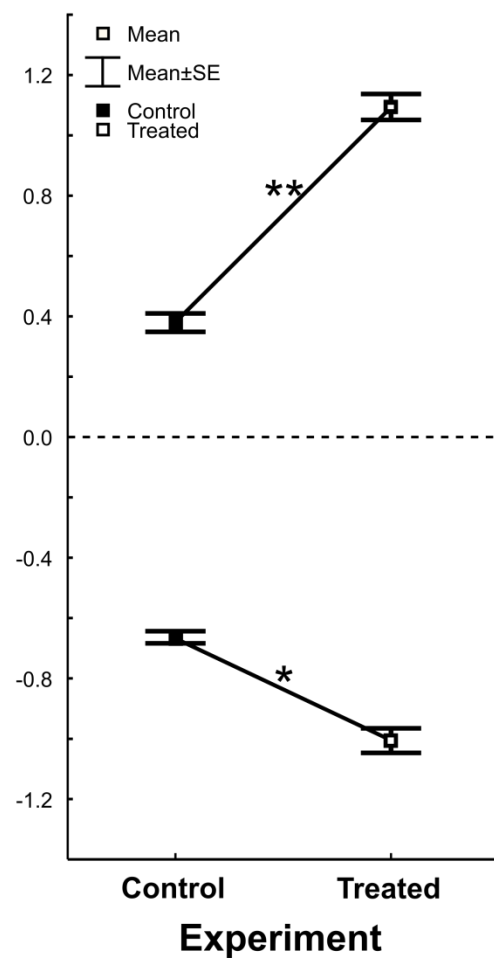
In order to further examine this possibility, we next analyzed the amplitudes of heteronymous dynamic reflexes over the entire range of background forces collected. These data are shown in Figure 4.7, with the corresponding mean  $\pm$  SE and the number of trials (n) used to calculate them listed in Table 4.3. There was a clear increase in the amplitude of facilitory heteronymous dynamic reflex force after reinnervation ( $p < 0.01$ , nested ANOVA with Tukey's HSD), and this increase was approximately three-fold. It was interesting to note that the amount of inhibition due to TA conditioning was also increased after reinnervation, although the significance of the difference between control and treated groups was much less ( $p < 0.05$ ;  $p = 0.38$  versus  $p = 0.008$ ). This dramatic increase in the amplitude of facilitory heteronymous reflexes due to antagonist stretch, taken together with the increase in the frequency of which facilitation occurs, suggests that there is a switch from net inhibition to net excitation in reciprocal stretch-evoked reflexes between antagonists after peripheral nerve regeneration. The remainder of this report is focused on further investigation of this phenomenon.

#### *Characteristics of inhibitory versus excitatory antagonist stretch reflexes*

Since the amount of heteronymous reflex force increased with the amount of background force experienced by G, this relationship was analyzed more carefully and compared between

**Figure 4.7 *Increased strength of both inhibition and facilitation after reinnervation.*** A:

Means and SEs of heteronymous dynamic reflex force (Heteron Dynamic Reflex) in G due to TA conditioning for individual experiments over a matched range of background forces in both control (white bars, n=5) and treated (gray bars, n=4) groups. Negative amplitudes represent inhibition, while positive amplitudes represent facilitation. Single asterisk denotes  $p < 0.05$ , double asterisk denotes  $p < 0.01$  (nested ANOVA with Tukey's HSD). B: Pooled data for control and treated groups for the data set in A for both facilitation (top) and inhibition (bottom). Connecting lines are for visual aid only.

**A****B**



**Table 4.3** *Heteronymous dynamic force due to reinnervated antagonist stretch.* G

heteronymous dynamic reflex force amplitudes due to TA conditioning from Figure 4.7.

Reflex amplitudes are reported as mean  $\pm$  SE and calculated from the number of trials (n)

identified, where one trial consists of a single pair of test and conditioned test responses.

Negative values represent inhibition. Asterisk denotes experiment in which the reinnervated

TA produced a homonymous reflex.

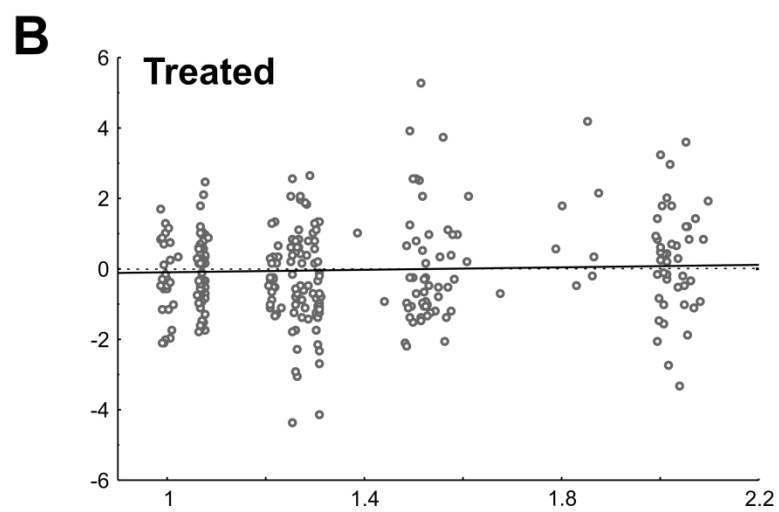
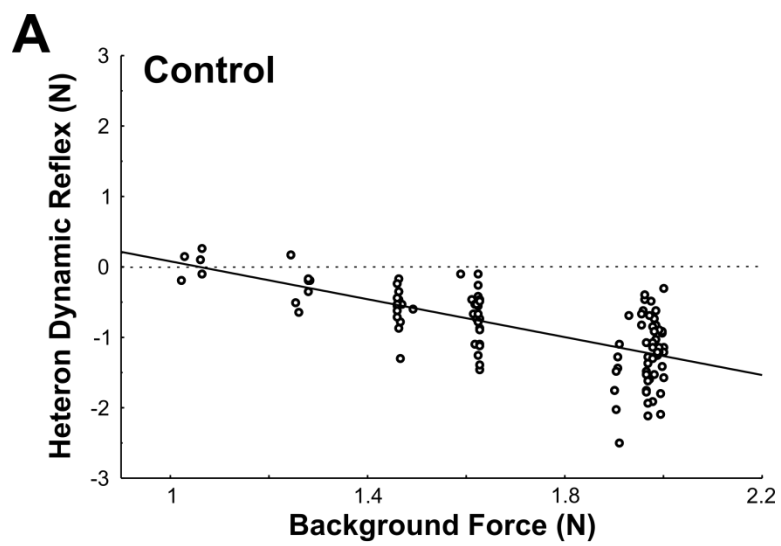
<b>Heteronymous Dynamic Reflex Force (N)</b>					
<b>TA conditioning of G: Inhibition</b>					
<b>Control</b>			<b>Treated</b>		
Exp	n	Mean $\pm$ SE	n	Mean $\pm$ SE	Exp
3	142	-0.5827 $\pm$ 0.0358	69	-0.8667 $\pm$ 0.0772	9
4	48	-0.6248 $\pm$ 0.0561	76	-0.8082 $\pm$ 0.0640	11
5	100	-0.9258 $\pm$ 0.0536	183	-1.0119 $\pm$ 0.0535	13
6	183	-0.7140 $\pm$ 0.0427	85	-1.2813 $\pm$ 0.1322	14
7	137	-0.5005 $\pm$ 0.0294	130	-1.4356 $\pm$ 0.0801	12*
Pooled	610	-0.6632 $\pm$ 0.0201	413	-1.0056 $\pm$ 0.0407	Pooled
<b>TA conditioning of G: Facilitation</b>					
<b>Control</b>			<b>Treated</b>		
Exp	n	Mean $\pm$ SE	n	Mean $\pm$ SE	Exp
3	25	0.5308 $\pm$ 0.966	156	110.91 $\pm$ 8.16	9
4	15	0.3205 $\pm$ 0.0436	120	86.99 $\pm$ 5.85	11
5	4	0.1747 $\pm$ 0.0341	162	103.49 $\pm$ 7.10	13
6	33	0.3556 $\pm$ 0.0488	142	133.68 $\pm$ 11.43	14
7	34	0.3417 $\pm$ 0.0411	15	77.26 $\pm$ 12.79	12*
Pooled	111	0.3796 $\pm$ 0.0304	580	1.0946 $\pm$ 0.0429	Pooled
<b>TA conditioning of G: No Change</b>					
<b>Control</b>			<b>Treated</b>		
Exp	n	Mean $\pm$ SE	n	Mean $\pm$ SE	Exp
3	76	0.0033 $\pm$ 0.0046	148	0.0042 $\pm$ 0.0030	9
4	24	-0.0100 $\pm$ 0.0097	16	-0.0143 $\pm$ 0.0133	11
5	25	0.0098 $\pm$ 0.0136	28	0.0032 $\pm$ 0.0110	13
6	71	-0.0093 $\pm$ 0.0066	25	0.0129 $\pm$ 0.0106	14
7	44	0.0191 $\pm$ 0.0079	2	-0.0908 $\pm$ 0.0058	12*
Pooled	240	0.0018 $\pm$ 0.0033	217	0.0037 $\pm$ 0.0030	Pooled

control and treated groups. An example of this relationship is shown in figure 4.8. All heteronymous reflex responses for a single experiment were plotted against background force and fitted with a linear regression line. In the control example (Figure 4.8A), there was a significant relationship between heteronymous reflex force and background force ( $r=-0.6851$ ;  $p<0.001$ , Pearson correlation). This plot indicates that not only is there an increase in the amplitude of inhibition with increasing G background force, but the frequency at which inhibition occurred also increased. These observations held true for 4 out of 5 control experiments. In treated animals, however, this relationship was completely lost. In the example shown in Figure 4.8B, it is clear that neither the amplitude nor the frequency of inhibitory heteronymous reflexes increased with G background force ( $r=0.0556$ ,  $p=0.303$ ).

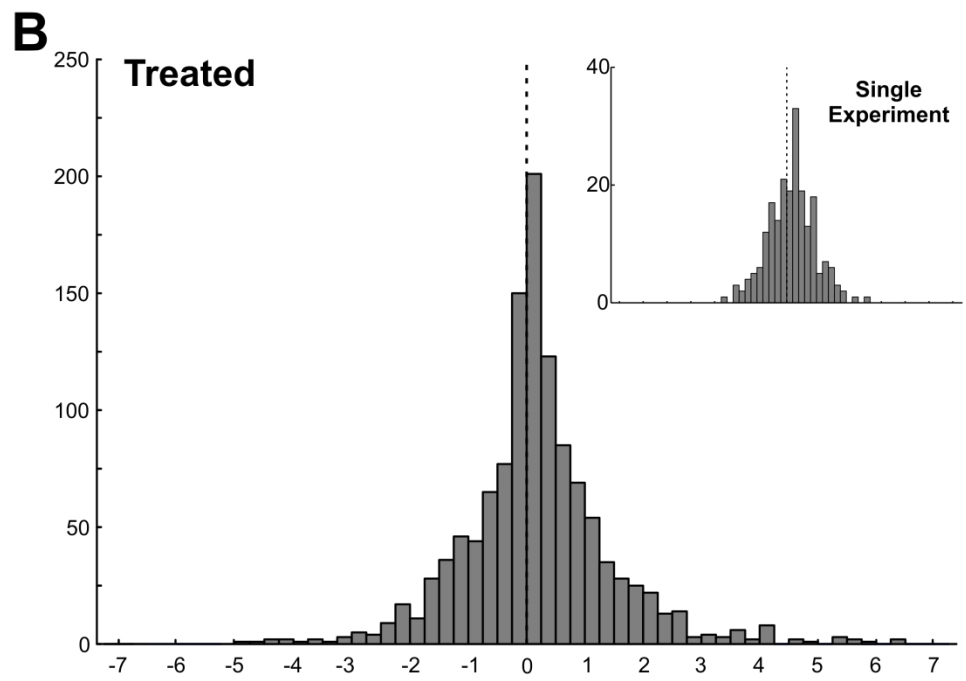
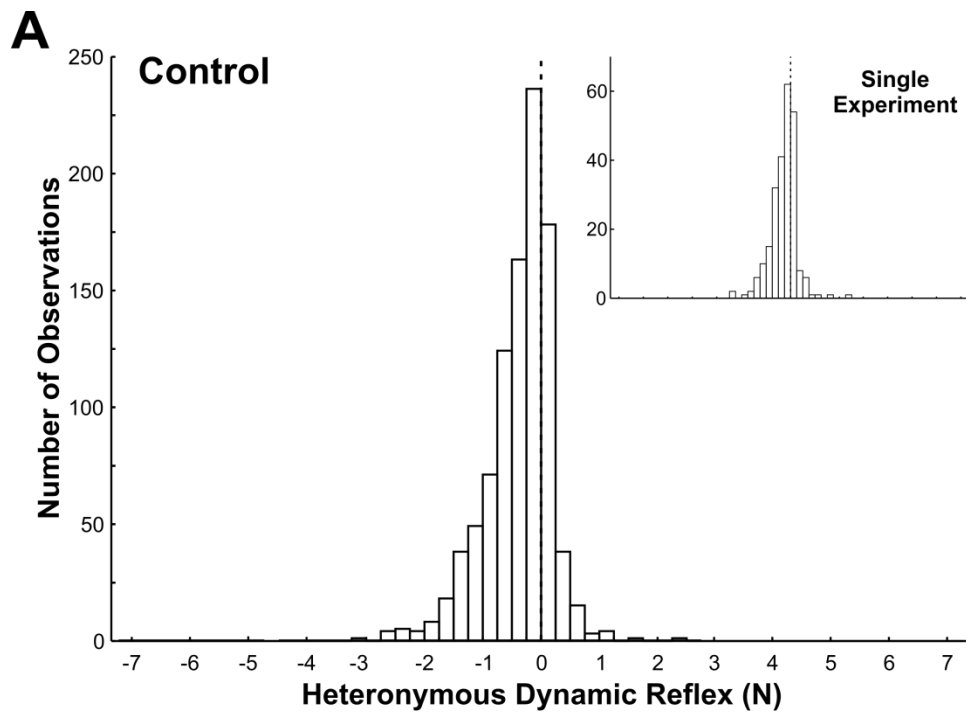
In order to further investigate the distribution of inhibition versus excitation due to stretch of the reinnervated TA, data were pooled per treatment group and histograms were generated in order to examine the overall frequency of heteronymous reflex amplitude occurrence. Only cases that were conclusively inhibitory or facilitory were included in this analysis.

Heteronymous reflex amplitudes were parsed into 0.25N bins and the distribution was compared between control and treated groups (Figure 4.9). We were then able to compute the statistical skewness of each group. Skewness is a measure of the asymmetry of the probability distribution around the mean. Therefore skewness with a negative value indicates that the majority of the data lies to the left of the mean, while skewness with a positive value indicates that the majority of the data lies to the right of the mean. In the control group, skewness was  $-0.8849 \pm 0.0789$ , indicating that the majority of heteronymous reflex responses were inhibitory. Skewness in the treated group was  $0.5357 \pm 0.0703$ , indicating that the majority of heteronymous reflex responses were facilitory. Furthermore, the distribution of heteronymous reflex amplitude is significantly different between control and

**Figure 4.8** *Relationship between heteronymous reflex and background force is absent after reinnervation.* Plot of heteronymous dynamic reflex amplitude (Heteron Dynamic Reflex) versus background force during quiescence. G force due to TA conditioning for single control (A) and treated (B) experiments. Linear regression lines are shown for each plot. Control  $r=-0.6851$ ,  $p<0.001$ ; Treated  $r=0.0556$ ,  $p=0.303$ . These findings were consistent for 4 out of 5 control animals and 4 out of 4 treated animals.



**Figure 4.9** *Distribution of heteronymous reflex amplitudes is unimodal.* Histogram of pooled heteronymous dynamic reflex amplitudes in G due to TA conditioning using 0.25N bins from all experiments over matched background ranges for Control (A) and Treated (B) groups. Control skewness =  $-0.8849 \pm 0.0789$ . Treated skewness =  $0.5357 \pm 0.0703$ . Distribution of heteronymous reflex amplitude is significantly different between control and treated groups ( $p < 0.001$ , Kolmogorov-Smirnov). Insets: Histogram for a single experiment from control (A) and treated (B) groups.

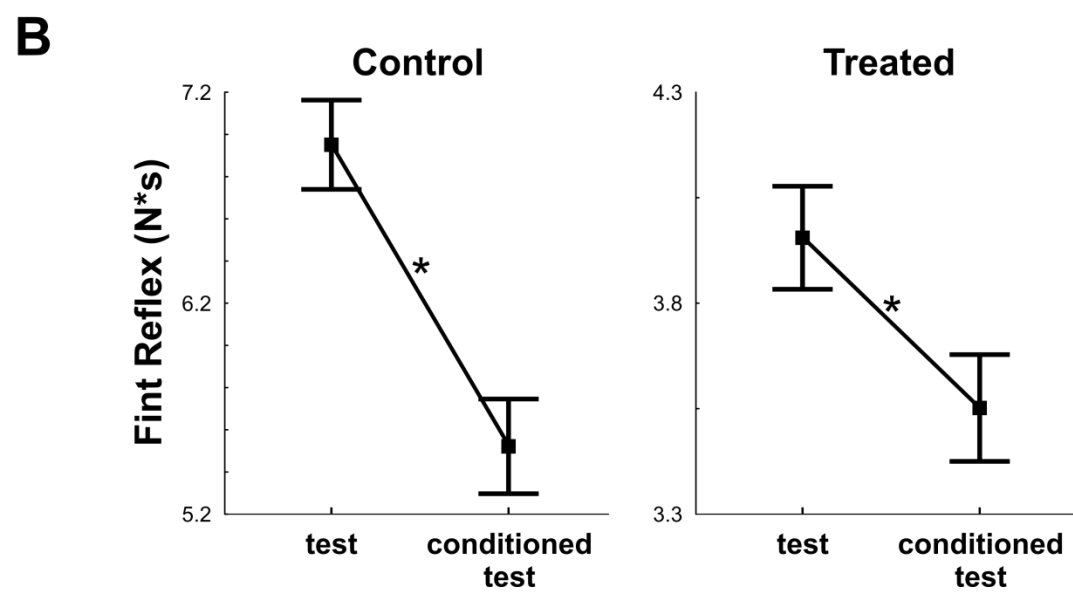
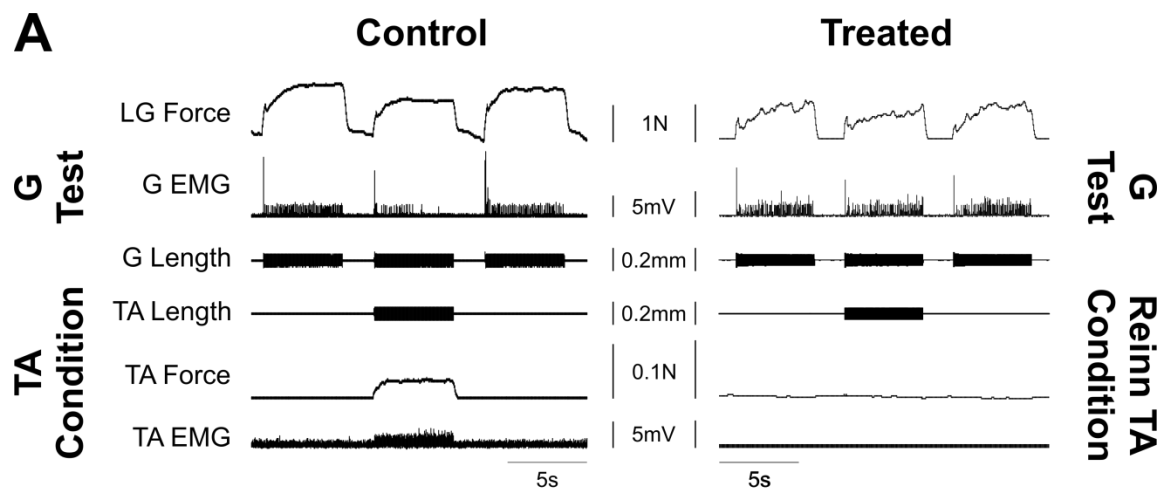


treated groups ( $p < 0.001$ , Kolmogorov-Smirnov). Perhaps even more striking is that the distribution of heteronymous reflex amplitudes was unimodal in both control and treated groups. This would suggest that the two pathways mediating inhibition and facilitation are activated simultaneously and the net summation of these pathways dictates the reflex response. If one pathway was selectively activated over the other, we would have expected two peaks in the distribution: one peak representing the inhibitory pathway and one peak representing the facilitatory pathway. Therefore although both inhibitory and facilitatory reflex pathways are active simultaneously, in controls the net output is balanced toward inhibition while in the treated group the net output is balanced toward excitation.

In order to gain further insight into the origin of facilitatory heteronymous reflexes due to antagonist stretch, muscles were subjected to vibration which should selectively activate Ia afferents (De-Doncker et al., 2003; Matthews, 1972). In control animals, the minimum background force required in order to produce appreciable vibration responses in either TA or G, varied widely, as did the amplitude of the force response. Therefore only a qualitative analysis of vibration responses could be performed. Regardless of background force, both muscles in the control group always produced vibration reflexes. In contrast, the reinnervated TA in the treated group never did. Using the same test-conditioned test paradigm used during ramp hold release stretch, we were able to qualitatively assess if either inhibition, facilitation, or both occurred in G in response to simultaneous vibration of TA. An example of this is shown in Figure 4.10. Force, EMG and length traces are shown for individual control (Figure 4.10A, left) and treated (right) animals at matched background forces. Vibration of TA almost exclusively produced inhibition of G in both animals.



**Figure 4.10** *Vibration produces inhibition of antagonists.* A: Isometric force, length, and EMG traces of G (test stimulus) and TA (conditioning stimulus) response to vibration from both a control (left) and a treated (right) animal at matched background forces (1N). Force traces have been filtered to remove vibration artifact and EMG has been rectified and integrated for clarity. The black bar under G force responses indicates it has been conditioned by TA. B-C: Area under the force response to vibration (Fint Reflex) was calculated and compared between test and conditioned test responses. Mean  $\pm$  SE for test and conditioned test responses for the same control (B) and treated (C) experiments shown above. Asterisk denotes  $p < 0.001$  (Student's t-test).

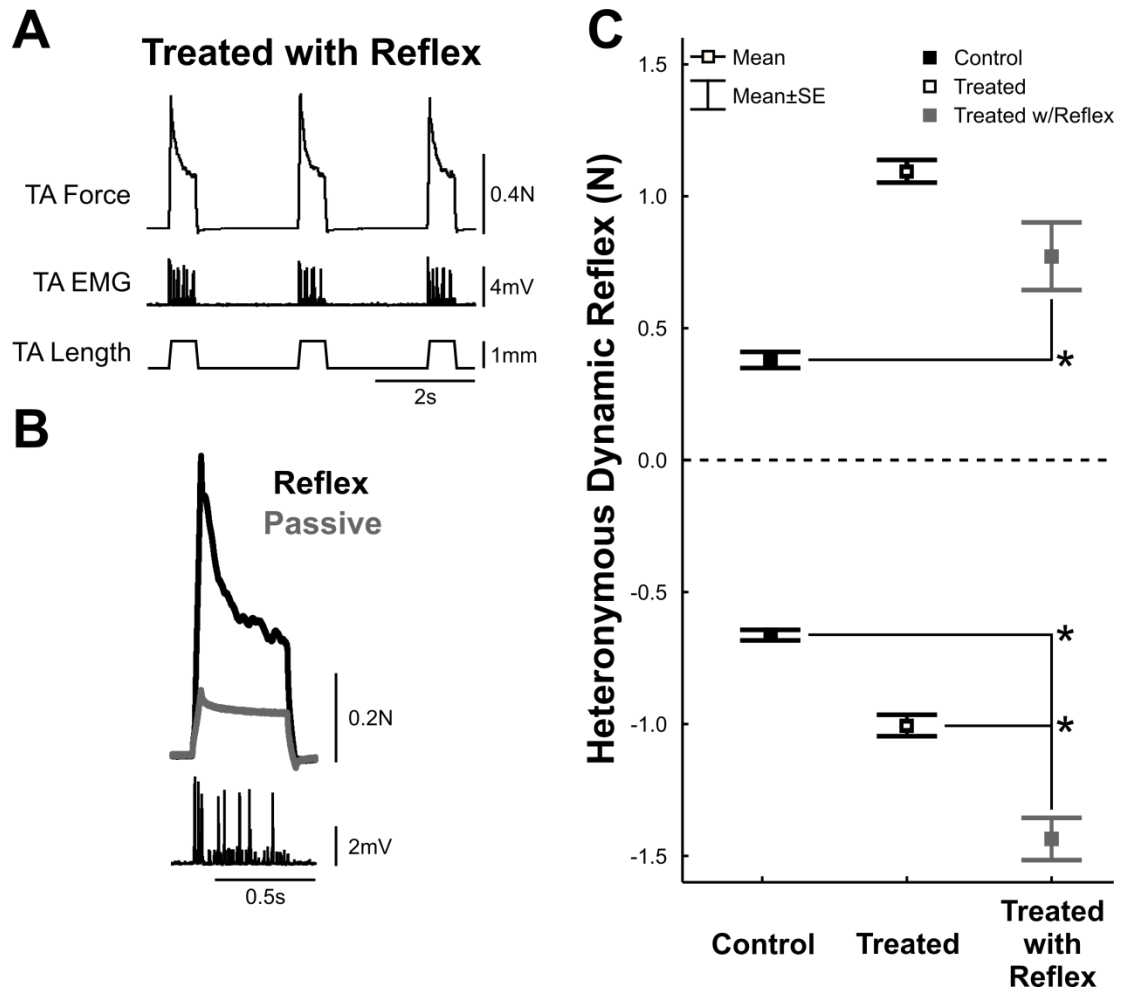


Furthermore, the differences in test and conditioned test responses were significant (Figure 4.10B). These results held true for all animals, regardless of treatment group. This may suggest that facilitation due to antagonist stretch is not mediated by Ia afferents.

*Reinnervated TA produced homonymous stretch reflexes in one case*

In one treated animal, the reinnervated TA produced a reflex response to homonymous stretch (Figure 4.11A-B). This was the first time our lab has ever observed this phenomenon. The nerve that was cut and surgically rejoined was identified and traced back to the TA muscle, indicating that the treated nerve did indeed send branches to the TA. Physiological verification of this innervation was not performed, however, so anomalous innervation could not be ruled out. Both the TA and G in this animal produced homonymous reflexes that were within the control range (Table 4.1). There was also no significant change in the distribution of inhibitory ( $p=0.0673$ , nested ANOVA with Tukey's HSD) versus facilitory ( $p=0.8563$ ) reflexes when compared to controls, although the incidence of inhibition was slightly increased. There were, however, significant changes in the amplitude of heteronymous reflexes elicited by the TA (Figure 4.11C, also see Table 4.3). The amplitude of facilitation was significantly increased above controls ( $p<0.001$ , ANOVA) but not above the rest of the treatment group ( $p=0.2996$ ). Interestingly, the amplitude of inhibition was greater than either controls or the rest of the treatment group ( $p<0.001$ ).

**Figure 4.11** *Reinnervated TA produced a homonymous stretch reflex in one case.* A: Raw isometric force, EMG and length traces in response to ramp hold release stretch for the single experiment in which the reinnervated TA produced a homonymous reflex. B: Isometric force and EMG (rectified, integrated, and filtered for clarity) traces from a reinnervated TA which produced a homonymous stretch reflex. Force response to ramp hold release stretch (black) is overlaid onto the passive (gray) response. Passive response did not produce EMG when stretched. C: Amount of facilitation and inhibition is increased when compared to control animals. Pooled means and SE of inhibitory and facilitory heteronymous stretch reflexes in control and treated groups (cf. Figure 4.7) compared to the single experiment in which the reinnervated TA produced a reflex (Reinn with Reflex, gray). Asterisks denote statistical significance  $p < 0.001$  (ANOVA). This case was significantly different from the control group in cases of facilitation and was significantly different from both control and the rest of the treated group in cases of inhibition.



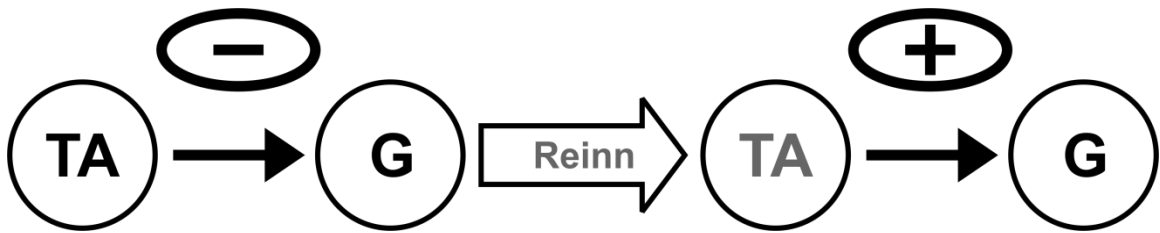
## Discussion

Our primary objective in this study was to examine the functionality of heteronymous projections of regenerated Ia afferents between antagonists, one of which was injury spared. We identified a number of factors that influence stretch reflex amplitude. Despite controlling for these factors, we found that reinnervated muscle produces heteronymous stretch reflexes in an injury-spared antagonist. However, the net effect changes from one of antagonist inhibition to antagonist facilitation (Figure 4.12). Both the frequency and amplitude of facilitatory heteronymous reflexes due to stretch of the reinnervated TA were increased. Our findings are therefore consistent with the retention of VGLUT1 contacts in regions of the spinal cord where interneurons that are known to receive primary afferent input are located (Alvarez et al., 2011). Furthermore, our findings support and give possible a mechanism for deficits in antagonist coordination during locomotion (Sabatier et al., 2011; Wasserschaff, 1990). Before we turn to a discussion of the ability of regenerated afferents to elicit stretch-evoked reflexes in antagonists, there are a few discrepancies between our data and pre-existing literature that must be addressed.

### *Reinnervated TA with homonymous reflex*

The presence of a homonymous reflex in a reinnervated TA was completely unexpected. This finding is despite numerous reports that reinnervated muscle fails to produce them in multiple species (Cope and Clark, 1993; Cope et al., 1994; Huyghes-Despointes et al., 2003; Haftel et al 2005; Maas et al 2007). Although the suture and neuroma from the initial surgery were visually identified and the nerve was traced back to the TA, innervation of the TA was not tested by direct stimulation of the nerve. Therefore it was entirely possible that there was

**Figure 4.12** *Redistribution of net antagonist effects after reinnervation.* Schematic of stretch-activated proprioceptive feedback between antagonist muscles before (left) and after (right) selective nerve injury and reinnervation. Solid arrows: antagonist heteronymous reflex. Minus and plus signs represent net inhibition and net excitation, respectively





an anomalous branch supplying the TA that was not cut during survival surgery. It was interesting that both TA and G homonymous reflexes were within control range but that the amplitude of heteronymous reflexes due to stretch of the reinnervated TA increased. Homonymous stretch reflexes were recently shown to increase after nerve crush (Prather et al., 2011). Therefore if part of the nerve supply remained uninjured and part consisted of regenerated nerves, the uninjured nerve supply could respond to injury in a similar way as they do for nerve crush. However, the homonymous reflex amplitudes were unchanged from controls. Furthermore, our findings in Chapter 3 indicate that injury-spared reflexes in muscle are actually decreased following synergist reinnervation. Therefore explanation of why heteronymous reflex amplitudes in this case are elevated remains elusive.

#### *TA response to G stretch*

The finding that stretch of G always produced facilitation of TA in controls during quiescence was also unexpected. This is in contrast to the normal reflex pattern in cats, where stretch of MG or SOL inhibits the TA (Nichols, 1989). During the active state, however, this inhibition changes to facilitation and is initiated by stretch of any of the triceps surae. We were unable to test antagonist stretch reflexes during the active state in our preparations due to the short length of crossed extension or sural reflex force responses and therefore cannot comment on the dependency of activity level in producing inhibition versus facilitation in our preparation. However, the difference during quiescence may be due to species differences in posture both at rest and during locomotion. In the rat, the limb is more collapsed on itself, with increased flexion at the knee and ankle. The knee and ankle may therefore require excitation of antagonists to maintain stability under these conditions.

The failure of G to produce heteronymous facilitory reflexes in TA suggests that there is still a post-synaptic component to stretch-areflexia. One important difference between these results and those discussed in Chapter 3 is the observation that these actions are mediated through either di- or trisynaptic pathways. On one hand, regenerated afferents seem to maintain connections with interneurons. On the other hand, regenerated motoneurons lose connections with some interneurons. This must necessarily be the case, as both the antagonist afferent and the interneuron are injury-spared. This observation would therefore clearly indicate that at least some post-synaptic component, such as the case with synaptic stripping, is involved at least in part in stretch-evoked reflex dysfunction after peripheral nerve regeneration. However, excitatory synapses from sources other than Ia afferents on regenerated motoneurons tend to recover better than inhibitory synapses (Alvarez et al., 2011; Brannstrom and Kellerth, 1999), and so our finding presents a bit of a conundrum. This is especially the case since these same regenerated motoneurons respond to other sources of excitatory input (cg. Figure 4.3). The question that remains is what causes regenerated motoneurons to lose synapses with some excitatory interneurons but retain them with others.

#### *Antagonist stretch reflexes initiated by regenerated afferents*

The presence of stretch-evoked antagonist reflexes elicited by regenerated afferents is most readily explained by the presence of VGLUT1 boutons in lamina V and VII after peripheral nerve regeneration (Alvarez et al., 2011). These are lamina in which primary afferents have been shown to make connections with interneurons that are either excitatory (Bannatyne et al., 2006; Bannatyne et al., 2009; Jankowska et al., 2009; Liu et al., 2010) or inhibitory (Hultborn et al., 1976b; Jankowska and Lindström, 1972), respectively. These interneurons are therefore likely candidates in mediating the observed facilitory or inhibitory antagonist

stretch reflexes. However, the identity of the facilitory reflexes unveiled here remain unknown. Available literature on polysynaptic excitatory pathways mediated by Ia afferents affecting antagonist motor pools is scarce. We do know that some of the interneurons located in lamina V and VII receive convergent input from both Ia and Ib afferents (Czarkowska et al., 1981; Jankowska et al., 1981; Fetz et al., 1979), but whether or not the interneurons involved in the reflexes we have observed are a separate population requires further study.

The observation that synaptic varicosities containing VGLUT1 were smaller would indicate that the amplitude of antagonist stretch reflexes may be decreased, but this was clearly not the case. Both facilitory and inhibitory reflexes were increased, with facilitory reflexes showing predominance. The anatomical redistribution of VGLUT1 after regeneration shows a 2.5 fold increase in the number of contacts in lamina V, where excitatory interneurons are located. This may suggest sprouting of regenerated primary afferents in lamina V. Central collateral sprouting of regenerated primary afferents has been observed on numerous occasions, especially in the dorsal horn (see Navarro et al., 2007 for review). Therefore an increase in the number of synaptic contacts on excitatory interneurons by regenerated afferents may explain the net increase in facilitation we observed here. This increase in synaptic contacts in lamina V must be interpreted carefully, as VGLUT1 in these lamina are representative of not only Ia afferents, but all primary afferents, including Ib's and cutaneous afferents (Alvarez et al., 2004).

The inhibition seen with vibration would, at first glance, seem to indicate that the observed facilitory responses are due to Ib afferents and not Ia's. However, Ib afferents are capable of responding to vibration, just not with one to one accuracy (Bullinger et al., 2011b; Matthews, 1993), and are normally more capable of responding at higher background forces. In order to

elicit vibration reflexes in these experiments, it was often necessary to stretch out the muscle to background force equivalents 4 or more times that of resting force. Therefore it was highly likely that Ib afferents were in fact activated under these conditions. It is also possible that this as yet unidentified facilitory reflex pathway may be trisynaptic. Thus vibration may not provide enough excitation through all three synapses to ultimately cause motoneuron firing. Ib afferents have previously been shown to make di- or trisynaptic connections with motoneurons through excitatory pathways (Bannatyne et al., 2006; Bannatyne et al., 2009; Jankowska et al., 2009), and a subpopulation of these interneurons also receives convergent input from Ia afferents (Czarkowska et al., 1981; Jankowska et al., 1981; Fetz et al., 1979). Therefore the finding of a lack of facilitory antagonist stretch reflexes with vibration must be interpreted with caution.

The observed increase in inhibitory amplitude of antagonist stretch reflexes, although small, was nonetheless significant. Alvarez et al. (2011) found a small decrease (approximately 20%) in the number of VGLUT1 contacts in lamina VII, where the interneurons mediating reciprocal inhibition are located (Hultborn et al, 1976b; Jankowska and Lindström, 1972). Furthermore, inhibitory synapses do not recover as well as excitatory synapses with reinnervation (Alvarez et al., 2011; Brannstrom and Kellerth, 1999). Therefore this increase presents a bit of a challenge to explain. It is possible that even though the total number of contacts in lamina VII remains the same, there is still a local redistribution of Ia synaptic contacts onto inhibitory interneurons. It is difficult to infer, again given the fact that VGLUT1 labels all primary afferents and is not selective for Ia afferents in any spinal location but lamina IX. It is also possible that the inhibitory pathways that remain are amplified in some way. Persistent inward currents act to amplify and prolong the effects of synaptic inputs through voltage-gated channels in motoneurons (Heckman et al., 2008;

Hultborn et al., 2004). In order for this mechanism to be valid, it would require enhancement of the persistent inward current at the interneuronal level since the target antagonist motoneuron is uninjured. Although heavily studied in motoneurons, their presence in interneurons remains unknown. It is also unknown how persistent inward currents would be affected by regeneration.

The question that remains is what causes the switch between facilitation and inhibition of antagonists after peripheral nerve regeneration? Our data suggest that this is not a switch per se, but a balance in the activity of two simultaneously active populations of interneurons. The net output of this interneuronal population would therefore be determined by the pathway that was stronger. If these two populations operated under a gating mechanism, we would have expected to see two peaks in our heteronymous stretch reflex amplitude histograms, one negative and one positive. Instead, the distribution was unimodal which suggests that the reflex amplitudes operate on a continuum. The simultaneous activity also seems likely given that primary afferents send projections to both excitatory and inhibitory interneurons that target the same motoneuron.

### *Summary*

In sum, the results presented here suggest that there is a profound reorganization of spinal circuits after regeneration. Although antagonist stretch-evoked reflexes are indeed present, this reorganization alters function such that it hampers the ability for antagonists to coordinate as they do normally. The switch from net inhibition to net excitation would cause antagonists to contract simultaneously. On the one hand, co-contraction would support stability at a joint (Enoka, 2008). On the other hand, it would not only impede efficiency and speed in alternating movements but may also impede normally coordinated antagonist

activity during locomotion or during correction of postural disturbances. The findings presented in this chapter may account for the increase in co-contraction between antagonists after reinnervation (Sabatier et al., 2011; Wasserschaff, 1990). Similar abnormal reflex coupling has been observed after stroke (Trumbower et al., 2010), spinal cord injury (Xia and Rymer, 2005), and other spastic conditions such as cerebral palsy (Kukke and Sanger, 2011). Although all three of these conditions certainly involve impairment in descending pathways, it seems likely that these changes profoundly influence the segmental spinal reflex circuits that underlie these actions. Therefore deficits in stretch reflex function are not limited solely to the presence or absence of Ia-motoneuron synapses after reinnervation; the synapses that do remain are reorganized in such a way as to further impair normal function. Several questions still remain. First, what is the identity of the interneurons mediating this increased facilitation? Second, what are the mechanisms behind the differential preservation of functional synapses after peripheral nerve regeneration?

## **CHAPTER 5: General Discussion**

Growing evidence suggests that central deficits remain after severed peripheral nerves regenerate, even after peripheral target reinnervation is largely successful. Therefore it is imperative that we understand the limitations of functional recovery so that better therapeutic strategies may be developed in order to overcome sensory and motor deficits that are a direct result of peripheral nerve injury. We have known for some time that reinnervated muscle fails to produce homonymous stretch reflexes. Current evidence suggests this areflexia is in part due to a retraction of Ia afferent collaterals from motor pools in lamina IX (Alvarez et al., 2011; Bullinger et al., 2011). This retraction from the entirety of lamina IX, along with a redistribution of synaptic contacts in lamina V and VII (Alvarez et al., 2011), suggest that other stretch-evoked reflex pathways mediated by regenerated Ia afferents may be expected as well. In order to understand the limitations of stretch reflex recovery after peripheral nerve regeneration, we carefully examined the ability of Ia afferents to generate heteronymous stretch reflexes among both synergist and antagonist motor pools, some of which were injury-spared.

Our results demonstrate a differential functional recovery of stretch reflexes after reinnervation, and this recovery closely parallels the redistribution in VGLUT1 contacts in the spinal cord (Alvarez et al., 2011). Thus we provide direct functional evidence that multiple stretch reflex pathways are disrupted. Furthermore, these results indicate that there is a profound discoordination of spinal reflexes and reorganization of spinal circuits after peripheral nerve regeneration. We found that regenerated Ia afferents are ineffective in

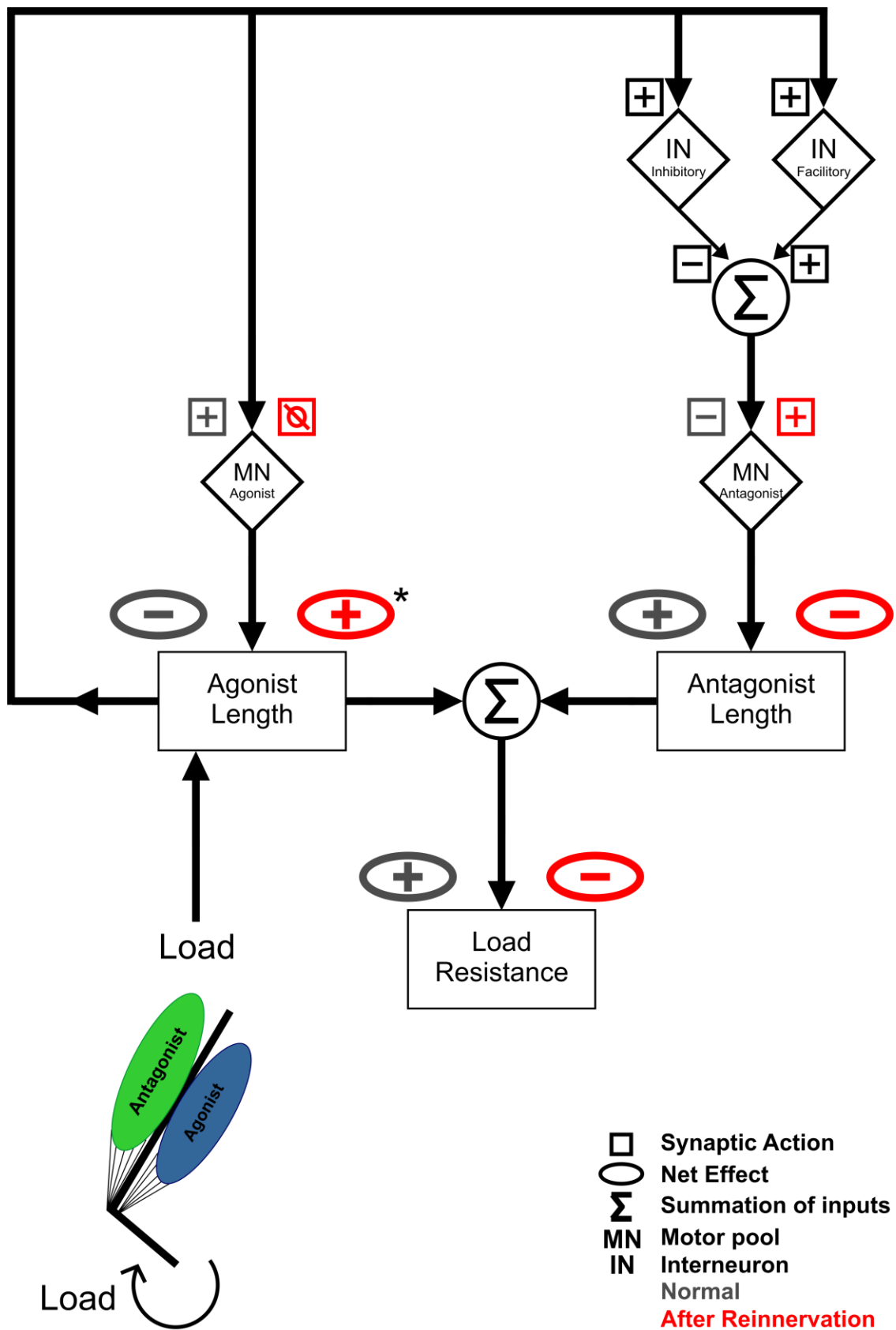
producing heteronymous stretch reflexes among synergists. Even synergist Ia afferents that are injury-spared produce decreased stretch in relation to reinnervation, suggesting that the process of reinnervation itself is adding insult to injury. To compound the problem even further, stretch reflexes in antagonist muscles switch from net inhibition to net facilitation.

The cumulative effects of these dysfunctional circuits are readily appreciated when one examines how they work together normally to compensate for unexpected perturbances, or increases in load. Normally, the stretch reflex works as a negative length feedback loop in regard to muscle length (Figure 5.1, gray boxes and ovals). An increase in load experienced at a joint stretches an agonist. Ia afferents then transmit this information into the spinal cord where they synapse with both homonymous and synergist motor pools. Increased activation of motor pools increases motor unit recruitment, which in turn causes the agonist to contract. Muscle contraction thereby shortens the agonist length (negative length feedback) so that it can resist the increase in load. However, these same afferents also project to antagonist motor pools through both inhibitory and excitatory interneurons. Normally, the sum of these interneuron outputs produces a net inhibition of the antagonist motor pools. This causes the antagonist muscle to relax, thus promoting muscle lengthening. Antagonist lengthening allows for the agonist muscle to quickly and efficiently resist the increase in load. In this way, length feedback acts as a corrective response in order to resist unexpected perturbances.

After peripheral nerve regeneration, the action of the entire feedback loop is reversed (Figure 5.1, red boxes and ovals). Since both homonymous and heteronymous stretch reflex actions are lost, Ia afferent activation fails to excite motor pools and the agonist muscle does not contract. Therefore this portion of the feedback loop now promotes muscle lengthening by allowing the muscle to be passively stretched due to the increased load. In other



**Figure 5.1** *Stretch reflexes change their net actions from load resistance to load assistance after peripheral nerve regeneration.* An increase in load increases the stretch of a muscle (agonist). Normally, this increased stretch decreases the length of itself through eliciting homonymous contraction while allowing antagonist muscles to lengthen. These two actions act together to resist the increased load. After reinnervation, the opposite occurs: agonists are allowed to passively lengthen with load and antagonists are shortened. These two actions taken together in effect assist the increased load. Note that the "+" net effect on length after reinnervation is the result of passive lengthening due to the lack of resistance, not active lengthening. See text for additional details. Squares: synaptic actions; ovals: net effects; MN: motor pool; IN: interneuron.



words, the resistance to passive stretch is decreased. This allows the limb to move in the direction of the load in a more uncontrolled way.

This problem is accentuated by the effects of regenerated afferents on antagonist muscle. This is because sum of the interneuron outputs now produces a net facilitation of antagonist motor pools. This causes the motoneurons to contract instead of relax, thereby shortening the muscle. Antagonist muscle shortening therefore actively moves the limb in the direction of the load. For example, let's say a friend hands a heavy box over to you. Normally, your limbs can resist the load through the feedback system discussed above and you are able to hold the box fairly level. After reinnervation, not only would you not be able to hold the box level, but the shortening of antagonist muscles would cause you to forcefully drop the box to the ground. Therefore the effects of peripheral nerve injury are twofold. First, recovery of stretch reflexes is limited. Second, the recovery that does occur exacerbates the problem.

Many motor deficits that have been observed to occur following either peripheral nerve regeneration or as a result of neurological disease can be attributed, at least in part, to similar abnormalities as we have just described. Locomotor changes associated with the loss of proprioceptive feedback have certainly been observed after peripheral nerve regeneration. Disruption of joint coordination (Abelew et al, 2000; Chang et al., 2009; Maas et al., 2007), as well increases in ankle yield under exacerbating conditions (Abelew et al, 2000; Maas et al., 2007) still persist. Furthermore, there is an increase in coactivation of antagonists during locomotion (Sabatier et al., 2011; Wasserschaff, 1990). However, dysfunctional coordination of muscular activity is also known to occur following certain disease states such as stroke (Trumbower et al., 2010), spinal cord injury (Xia and Rymer, 2005), and cerebral palsy (Kukke and Sanger, 2011). The result of this dysfunction is often loss of independent control

of joints, decrease in the ability to provide gravitational support to the limb, and reduced speed in generating volitional movements. It is interesting to note that the common denominator in all three of these disease states is an increase in spasticity.

In sum, the data presented here suggest that limitations on functional recovery of spinal circuits after peripheral nerve regeneration extend far beyond circuits that were directly injured. Some of the limitations described can be attributed to a disconnect between Ia afferents and motoneurons through afferent retraction, but the mechanism behind this retraction remains unknown. The mechanism by which some synapses are differentially retained, e.g. those mediating antagonist reflexes, also remains unknown. Even though this retention resulted in abnormal reflexes, on the one hand this mechanism could be exploited to promote afferent regrowth in order to reform synapses that are permanently lost. On the other hand, it is highly likely that even if regrowth of these afferents into areas of the spinal cord where synaptic loss occurs is achieved, the functional output of those successful contacts will be abnormal. The data presented in this report provide the physiological evidence necessary to justify moving forward with studies investigating the mechanisms by which these changes occur.

## **APPENDIX A: Commonly used abbreviations**

Ia	Group Ia primary afferent
Ib	Group Ib primary afferent
AHP	Afterhypolarization potential
EPSP	Excitatory post synaptic potential
eSSP	Excitatory stretch evoked synaptic potential
G	Gastrocnemius (both medial and lateral heads)
GTO	Golgi tendon organ
IPSP	Inhibitory post synaptic potential
iSSP	Inhibitory stretch evoked synaptic potential
LG	Lateral gastrocnemius
LGS	Lateral gastrocnemius-soleus
MG	Medial gastrocnemius
TA	Tibialis anterior

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